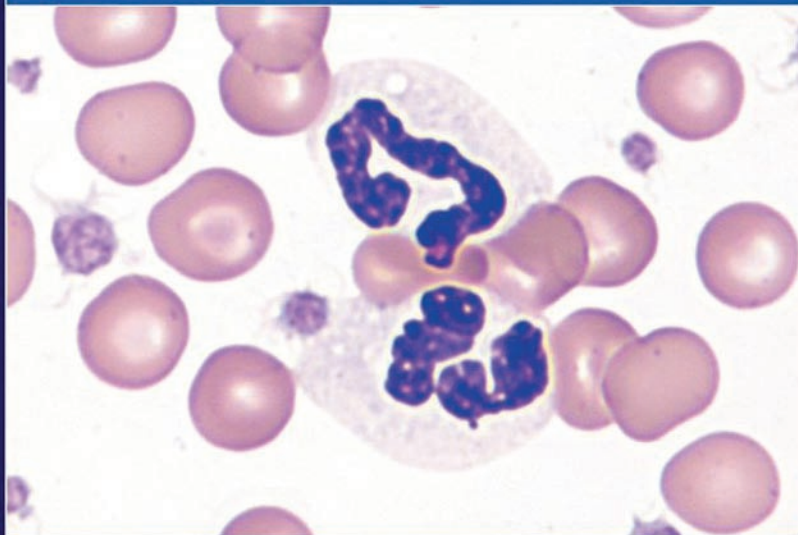


SCHALM'S
**VETERINARY
HEMATOLOGY**

SIXTH EDITION



EDITED BY: DOUGLAS J. WEISS & K. JANE WARDROP

 WILEY-BLACKWELL

SCHALM'S

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SIXTH EDITION

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DEDICATION

To those veterinary professionals who made the critical observations and performed the key experiments that provided the intellectual foundation for the discipline of Veterinary Hematology. Whether or not their names appear as authors in this text they have contributed to the concepts presented herein and have inspired those of us whose names appear.

Douglas J. Weiss

To my friends and colleagues for their continual support, and to those mentors who have nurtured my interest in clinical and investigative hematology. I am indeed fortunate to be able to work in an area that both challenges and rewards me.

Also dedicated to my family: Marv, Jessica, Michael, and Evan, for bringing such joy and balance into my life.

K. Jane Wardrop

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PREFACE TO THE SIXTH EDITION

The sixth edition of *Schalm's Veterinary Hematology* has undergone significant updating, reorganization, and refocusing. Several global changes have been made with the goal of making the information more accessible and improving cohesiveness and readability of the text. First, we grouped topics within established disciplines in hematology. Secondly, we introduced informative chapter outlines at the beginning of each chapter and extensively refer the reader to other chapters within the text. Finally, we edited the text in an attempt to make the presentation as clear and uniform as possible.

In addition to organizational changes, we added two new sections; Hematotoxicity and Quality Control and Laboratory Techniques. The Hematotoxicity section was thought necessary because of the large number of clinical pathologists now involved in preclinical safety assessment and toxicology and because of present concerns about environmental toxicants. In that regard, we also expanded the laboratory animal hematology chapter in the book. The Quality Control and Laboratory Techniques section was added for several reasons. First, this section acknowledges and supports the growing group of veterinary laboratory technicians. Secondly,

this section may help to standardize testing and quality control in clinical and industrial laboratories. Thirdly, this section may support and encourage research in veterinary and comparative hematology.

We have also expanded other sections in the book. The Hematologic Neoplasia section was extensively reorganized using the World Health Organization classification system as the basis for classification of leukemias and lymphomas. This expansion reflects the dramatic progress made in this discipline in recent years. The Species Specific Hematology section has also been expanded by increasing the number of photographs of blood cells and by adding discussions of bone marrow where appropriate. This should be helpful when veterinary clinical pathologists encounter blood or bone marrow preparations from species with which they are not familiar.

We hope that you will find the many changes made in this edition to be beneficial. We thank the previous editors, section editors, and authors for their wisdom, knowledge, time, and commitment.

Douglas J. Weiss
K. Jane Wardrop



SECTION I

Hematopoiesis

Maxey L. Wellman

Embryonic and Fetal Hematopoiesis

KELLI L. BOYD and BRAD BOLON

Basic Principles of Hematopoietic Development
 Cell Structure and Function
 Primitive Hematopoiesis
 Erythroid Cells
 Other Cells

Definitive Hematopoiesis
 Hemoglobin Switching
 Molecular Mechanisms Regulating Hematopoietic
 Development

Acronyms and Abbreviations

AGM, aorta-gonad-mesonephros; Bmp, bone morphogenetic protein; 2,3-DPG, 2,3-diphosphoglycerate; E#, day of embryonic development, where the number indicates age of the embryo in days after conception; EPO, erythropoietin; fL, femtoliter; Gata1, 2, and 4, GATA-binding proteins 1, 2, and 4; HSC, hematopoietic stem cell; Ihh, Indian hedgehog; IL, interleukin; P#, day of postnatal development, where the number indicates age of the neonate in days after delivery; pg, picogram; PU.1, purine box-binding transcription factor; Scl/Tal-1, stem cell leukemia/T-cell acute leukemia factor.

The complexities of hematopoietic system development have been highly conserved throughout vertebrate evolution. Understanding the embryonic and fetal origins of hematopoiesis provides important insights regarding the function of the adult hematopoietic system. Hematopoiesis in embryonic and fetal animals has been studied intensively for several decades as a model for hematopoietic progression in humans. Recent technical advances have allowed researchers to characterize the spatial and temporal relationships as well as the cellular and molecular mechanisms of hematopoietic development.

This chapter reviews the basic biology of hematopoietic development in the mouse (*Mus musculus*). This appraisal will emphasize hematopoietic events during the embryonic and fetal stages of development, but also will cover selected features of neonatal hematopoiesis.

BASIC PRINCIPLES OF HEMATOPOIETIC DEVELOPMENT

Cell Structure and Function

Blood cells produced at different stages of development differ in morphology and function. Thus, primitive (“fetal”) cells fabricated early in gestation have mark-

edly different properties from their definitive (“adult”) counterparts produced during late gestation and in postnatal life. This principle has been characterized most completely in erythroid lineage cells. Primitive erythrocytes (RBCs) are formed in the yolk sac, whereas definitive RBCs are produced by the liver and later spleen and bone marrow. Primitive RBCs are nucleated in circulation until approximately day 12.5 (E12.5) of gestation, after which nuclei gradually become condensed before being shed between E14.5 to E16.5.³⁵ Enucleated primitive RBCs retain their large size and can remain in circulation until as late as postnatal day 5 (P5). Both primitive and definitive RBCs are released during most of the latter half of gestation (E10 to E18), although the ratio shifts as time progresses from mainly primitive to mainly definitive RBCs.

Primitive and definitive RBCs can be distinguished by their size. The volume of primitive RBCs varies from 465 to 530 femtoliters (fL) which is approximately six times larger than that of definitive RBCs.³⁵ The hemoglobin content of primitive RBCs, 80 to 100 picograms (pg)/cell, also is nearly six times the amount found in definitive RBCs.³⁵ Both primitive and definitive RBCs have basophilic cytoplasm when first produced due to abundant rough endoplasmic reticulum, but basophilia recedes as maximal hemoglobin content is achieved.

Other hematopoietic lineages also differ in cell structure and function during the course of development. Primitive megakaryocytes from the yolk sac contain fewer nuclei of lower ploidy, are about half the size, and respond differently to cytokine stimulation relative to definitive megakaryocytes.⁴⁷ Primitive macrophages that originate in the yolk sac⁴² lack certain enzyme activities, are capable of division, and survive for extended periods compared to definitive monocyte-derived macrophages. These functional differences are related to the roles that the two cell populations appear to play. Primitive macrophages are the source for many tissue macrophages in embryonic through juvenile stages of development, whereas definitive macrophages are the source for circulating monocytes and resident macrophages characteristic of the adult immune system.

PRIMITIVE HEMATOPOIESIS

The processes that drive primitive and definitive stages of hematopoiesis as well as the events that regulate transition between the two stages are mediated by a constellation of factors.^{1,30,45,46} Cell adhesion factors, growth factors, and transcription factors that participate in this process often support differentiation of multiple hematopoietic cell types,^{10,29,36} and the dependence of a given cell lineage on any particular molecule may differ between primitive and definitive hematopoiesis.

Erythroid Cells

Hematopoiesis occurs at multiple sites within the embryo and in extraembryonic tissues. The first phase of blood cell production, referred to as *primitive hematopoiesis*, is responsible for producing blood elements during the earliest stage of embryogenesis. Primitive hematopoiesis takes place in the visceral yolk sac beginning at approximately E7.0.^{15,34} Thus, primitive hematopoietic cells are among the earliest distinct tissues to differentiate in the embryo. Formation of primitive cells declines rapidly after E11. The visceral yolk sac or extraembryonic splanchnopleure (the term for a structure in which mesoderm and endoderm are directly apposed) arises from the migration of extraembryonic mesoderm streaming from the caudal primitive streak along the inner surface of the visceral endoderm. The mesodermal cells committed to initiate and support hematopoiesis have been termed *hemangioblasts* because the contiguity of primitive hematopoiesis and vasculogenesis in both space and time suggests that primitive hematopoietic and endothelial cells in the yolk sac share a common ancestor.^{1,9} Hemangioblasts arise as undifferentiated cells at the primitive-streak stage and commit to producing a particular cell lineage before blood island formation.^{34,44} These pluripotent cells also can differentiate into other mesenchyme-derived tissues.

Between E7.5 and E9.0, hemangioblasts form multiple aggregates termed *blood islands*.³⁵ Each blood island contains a central core of unattached inner hemangioblasts

(hematopoietic progenitors) surrounded by a rim of spindle-shaped outer hemangioblasts (endothelial progenitors).¹⁵ Nucleated erythroid cells are first recognized in the cores of the blood islands at E8.0 and are evident circulating in the cardiovascular system starting at E8.25.¹⁸ At this stage embryonic erythroblasts enter the circulation, where they continue to divide until approximately E13.0.

The majority of cells produced during primitive hematopoiesis are of the erythroid lineage. Committed erythroid colony-forming cells arrive in the yolk sac at approximately E7.25. These cells expand until E8.0 and then differentiate into primitive erythroblasts; all colony-forming cells have regressed completely by E9.0,³⁴ which corresponds approximately to the earliest phase of definitive erythropoiesis. Primitive erythroblasts serve as the sole source of RBCs in the early embryo from E8.0 to approximately E10.5³⁴ and remain an important source of RBCs until E13. Thus, embryos with a developmental age between E8.0 and E11 that are anemic suffer from a defect in primitive erythropoiesis.^{26,38} Interestingly, seemingly profound defects in primitive hematopoiesis leading to persistent functional defects in adulthood may not elicit an aberrant hematologic profile in the embryo.

Other Cells

Recent studies suggest that other hematopoietic cell lineages also are generated in the yolk sac during this primitive stage of hematopoietic development. Primitive lymphoid precursors and even some adult stem cells evolve at E7.5 and subsequently seed other sites of hematopoiesis, including the aorta-gonad-mesonephros region (AGM), umbilical vessels, and liver.⁴⁰ Primitive macrophages have been identified in the yolk sac by E8.0⁴ to E9.0.³⁴ In vitro experiments have demonstrated that E7.5 yolk sac cells can give rise to functional megakaryocytic precursors by E10.5.⁴⁷ Many hemangioblasts actually serve as bi- or oligo-potent progenitors, including those capable of commitment to erythrocytic/myeloid,⁴ erythrocytic/megakaryocytic,²⁷ granulocyte/macrophage,³⁴ and lymphoid (B cell and T cell)/myeloid lineages. Stem cells for mast cells have also been reported to arise in the yolk sac during primitive hematopoiesis.³⁴

DEFINITIVE HEMATOPOIESIS

The second stage of blood cell production, termed *definitive hematopoiesis*, is thought to arise primarily from the AGM.^{3,27} The AGM is an amorphous band of intraembryonic splanchnopleure that encompasses the dorso-medial wall of the abdominal cavity. The AGM domain is the main source of mesenchyme-derived, definitive hematopoietic stem cells (HSCs) that will serve the developing animal during late gestation and post-natal life. Initiation of definitive hematopoiesis ranges between E8.5 and E9.25, with definitive HSCs evident in the AGM by no later than E10. Peak production of

HSCs in the AGM occurs between E10.5 and E11.5, at which time they comprise almost 10% of all AGM cells. Although controversial, some AGM-independent HSCs may also arise from the allantois, chorion, definitive placenta, umbilical arteries, and yolk sac. The actual contribution of these secondary sites to the overall HSC population has yet to be defined. However, the placenta appears to serve a particularly important role. The yolk sac also appears to be an essential secondary site because it is a source of multiple progenitor cell lineages and remains for at least a day after the AGM has halted HSC production.²⁸

Regardless of their original site of de novo synthesis, HSCs migrate to seed other locations that support definitive hematopoiesis: embryonic liver, followed by embryonic thymus, fetal spleen, and bone marrow (in that order). These latter destinations do not produce HSCs de novo but rather contain niches suitable for expansion of newly arrived HSCs.³³ The suitability of such niches is controlled by specific characteristics of their stromal support cells.³³ The embryonic liver is colonized first, apparently because it shares many molecular and functional similarities with the yolk sac.³¹ It provides the major locus for definitive hematopoiesis from E12 to E16.³⁹ The HSCs enter the embryonic liver in several succeeding waves between E9.0 or E10.0 and E12.¹² The first HSCs to enter the liver are pluripotent and can form any type of hematopoietic cell. Their first step in intra-hepatic maturation is to commit to a more limited range of lineage options, typically as either an erythromyeloid progenitor or a common myelolymphoid progenitor.²² Definitive erythroid precursors mature and become enucleated within erythroid islands in the liver before entering the circulation.²⁷ Liver-derived myelolymphoid progenitors subsequently develop into bi-potent cells (B cell and myeloid, or T cell and myeloid) before committing to produce a single cell lineage.²² Some T cell progenitors have a bi-potent commitment to NK cell lineage. T cell precursors destined for transfer to the embryonic thymus are produced even in athymic mice, indicating that the fetal liver may play a role in promoting early T cell differentiation.^{20,21}

Embryonic thymus and fetal spleen are seeded either from the liver or AGM, or both, beginning about E13 for thymus and E15 for spleen. The thymus typically accepts only those HSCs that are committed to make T cells, whereas other multi-potent myelolymphoid elements are directed to other sites.²⁰ The number of T cell precursors in liver is abundant at E12 but decreases thereafter, whereas the population of intra-hepatic B cell progenitors exhibits a reverse trend.¹⁹ Most types of definitive hematopoietic cells in the spleen arise from precursor cells that commit to a specific lineage before leaving the liver. Multi-potent HSCs entering the spleen cease proliferating and differentiate into mature macrophages. These cells may regulate intra-splenic erythropoiesis.

The bone marrow first receives HSCs from hepatic depots at about E16.^{39,45} Thereafter, the allocation of colony-forming hematopoietic precursors shifts from a

primarily hepato-centric localization at E18 through a more dispersed distribution (bone marrow, liver, and spleen in approximately equal numbers) at P2 to a profile favoring bone marrow and to a lesser extent spleen at P4 and after.⁴⁹ Thus the bone marrow, liver, and spleen function cooperatively to regulate definitive hematopoiesis. While cooperating, each organ supports a molecularly distinct subset of hematopoietic progenitors.

Committed hematopoietic progenitors necessary to foster all lineages observed in adult animals arise during definitive hematopoiesis. The AGM-derived HSCs contribute to all major hematopoietic cell lineages. The HSC population from the placenta reportedly supports the genesis of erythroid, lymphoid (both B cell and T cell lineages), and myeloid elements. By comparison, the lineages sustained by yolk sac-derived HSCs are limited to lymphoid and myeloid cells.⁴⁰ Whether or not progenitors for a given definitive cell lineage arising from distinct HSC populations exhibit different functional and molecular properties during late fetal and/or postnatal life has yet to be determined.

Late-stage embryos (E13 to E15), fetuses (E16 to birth), and neonates which present with anemia are afflicted with a defect in definitive erythropoiesis. Abnormalities associated with this presentation include the total absence of definitive hematopoiesis,^{25,41} and an inability of progenitor cells to properly colonize intra-embryonic sites of hematopoiesis. Multiple cell lineages may be affected; such a combined effect suggests that the hematopoietic defect occurs in a bi- or multi-potent stem cell rather than in one committed to forming a specific cell lineage.⁴³ Presentation with late-stage anemia also might result from a general delay in growth and development rather than a focused anomaly in the erythrocytic lineage.⁷

Young animals have circulating blood cell numbers that are different from adults.³⁹ RBC numbers more than double between birth and young adulthood. Circulating leukocyte counts at birth are approximately 20% of adult levels before increasing to adult numbers by 6 to 7 weeks of age. Platelet counts in neonates are approximately one-third numbers.

HEMOGLOBIN SWITCHING

Primitive and definitive RBCs bear a battery of seven α - and β -globins, the mix of which varies with the developmental stage. The α -globins are encoded by three genes (ζ , α_1 , and α_2), whereas β -globins are encoded by four main genes ($\epsilon\gamma$, $\beta H1$, βmin , and βmaj). The globins of a given type (e.g. α - or β -globins) typically exist as a series of closely linked homologous genes and related pseudo-genes located on the same chromosome;^{16,24} mouse globin genes are carried on chromosomes 7 (β -globins) and 11 (α -globins). All seven mouse globin genes are transcribed during erythroid development, but the production of three— ζ , $\epsilon\gamma$, and $\beta H1$ —is limited to primitive RBCs.²³ A consequence of this limitation is that mouse β -globin genes, although

closely related to human globins in most respects, do not follow the human pattern of up-regulation in the sequence of their chromosomal arrangement.^{23,24}

The extent of individual globin gene expression and the blend of globin genes that are expressed vary over time. For example, enucleated primitive RBCs contain relatively more β_{min} than do definitive RBCs. At E11.5, β_{min} constitutes approximately 80% of the β -globin in circulation. This level is reduced by approximately 60% at birth. Primitive RBCs express increasing levels of adult globins as gestation progresses, whereas definitive RBCs harbor only the adult protein variants. This evolution indicates that the pattern of globin expression switches as the primitive RBCs are replaced by definitive RBCs. Molecular mechanisms which regulate the switching process are complex.¹⁷ The timing of this switch, between E10.5 and E12.5, coincides with the initial escalation in definitive erythropoiesis. Perturbed timing of this switch is a feature of some murine models of hematopoietic disease.⁶

Successful maintenance of the developing conceptus depends on preferential capture of oxygen in embryonic and fetal tissues. Therefore, primitive RBCs generally have a higher affinity for oxygen than do maternal RBCs, although domestic cats are an exception. This sequestration of oxygen is mediated by two primary mechanisms. The mechanism pertinent to the early embryonic period is the greater affinity of embryonic hemoglobin in primitive RBCs for oxygen relative to that of adult hemoglobin.² Alternatively, definitive RBCs in the late embryo and fetus possess a lower concentration of 2,3-diphosphoglycerate (2,3-DPG) than do maternal RBCs. Higher levels of 2,3-DPG facilitate oxygen release into tissues. After birth, levels 2,3-DPG content of RBCs rise to adult levels within 10 to 15 days.

MOLECULAR MECHANISMS REGULATING HEMATOPOIETIC DEVELOPMENT

A wide spectrum of growth factors, hormones, and transcription factors are required to specify the various stages of hematopoietic development in mammals. The entire meshwork responsible for directing any given event has not been completely characterized. Shifting levels of several transcription factors have been shown to modify blood cell production. Insufficiencies in many of these molecules act by forestalling primitive hematopoiesis in the yolk sac. For example, genesis of erythroid precursors is impacted by deficits in GATA-binding protein 1 (Gata-1),¹³ shown in vivo to prevent erythroid maturation; Gata-2,⁴⁸ demonstrated in vivo to abort precursor expansion; and Gata-4,⁵ for which an in vitro shortage thwarts hemangioblast-mediated specification of blood islands and their associated vessels. These effects occur because the GATA consensus elements are critical regulatory regions in many erythroid-specific genes. All cell lineages are affected by stem cell leukemia/T-cell acute leukemia factor 1 (Scl/Tal-1).³⁸ Abnormal levels of transcription factors can also act later in gestation to disrupt definitive hematopoiesis.

For example, purine box-binding transcription factor 1 (PU.1) is required for production of definitive (monocyte-derived) macrophages but not their primitive (yolk sac-derived) counterparts. This disparity in response is intriguing in that PU.1 is highly expressed during early hematopoiesis but fluctuates in various cell lineages as time progresses.¹⁰ Normal genesis of many progenitor cells, including bi-potent erythroid/megakaryocytic progenitors as well as B cell and T cell progenitors, requires that PU.1 levels be reduced, whereas production of myeloid progenitors necessitates an increase in PU.1.¹⁰

Secreted molecules also are important regulators of hematopoietic development during gestation. For example, erythropoietin (EPO) sustains both primitive and definitive erythropoiesis by stimulating proliferation and differentiation of immature primitive and definitive RBCs.²⁵ Reduction in EPO activity within the yolk sac greatly reduces the number of colony-forming cells and erythroblasts via excessive apoptosis. Thrombopoietin fulfills a similar function for megakaryocytes, although other cytokines (interleukin-3 [IL-3], IL-6) and growth factors (granulocyte-colony stimulating factor, stem cell factor) also are required.⁴⁷ Other ligand/receptor signaling pathways shown to affect hematopoietic development include the endoderm-derived molecule Indian hedgehog (Ihh)⁸ and bone morphogenetic protein 4 (Bmp4),¹¹ both of which participate in blood island production and vasculogenesis in the yolk sac. In general, secreted molecules act via their interaction with a specific transcription factor.

Cell adhesion molecules of the integrin family are essential for the proper migration of hematopoietic precursors. For instance, β_1 -integrins are essential if HSC are to reach the embryonic liver, and later the fetal spleen and bone marrow, at the appropriate developmental stages.³⁷ A loss of β_1 -integrins prevents adhesive interactions between HSCs and endothelial cells, thereby stranding the HSCs within vessels.³² Some integrins have functions in addition to their targeting role. For example, β_4 -integrins are required not only for correct homing but also for expansion and differentiation of erythroid and B cell precursors in liver, spleen, and bone marrow. As with secreted factors, the activities of some integrins relate more to late gestation and neonatal stages rather than earlier stages of hematopoietic development. This chronology has been documented for β_4 -integrin with respect to lymphoid and myeloid differentiation.¹⁴

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Structure of Bone Marrow

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Supporting Structures
Vasculature and Sinus Architecture
Innervation

Cellular Organization
Megakaryocytes
Erythroblastic (Rubriblastic) Islands
Granulocytes
Lymphoid Cells and Macrophages
Stem Cell Niches

Acronyms and Abbreviations

HSC, hematopoietic stem cell; RBC, red blood cell; WBC, white blood cell.

Bone marrow is the major hematopoietic organ in adults, and a primary lymphoid organ. Bone marrow is a diffuse organ which constitutes approximately 3% of the body mass in rats, 2% in dogs, and 5% in humans.³⁹ Marrow tissue is present in the central cavities of axial and long bones, and consists of a sinusoidal system, hematopoietic cells, adipose tissue, supporting reticular cells, and extracellular matrix. The complex vasculature and rich innervation of the marrow reflect the multiplicity of signals contributing to the control of hematopoiesis. Bone marrow is a dynamic organ capable of structural and functional remodeling in response to nutritional factors, endocrine signals, and variations in demand for the production of red blood cells (RBCs), white blood cells (WBCs), and platelets. This chapter will review the structure of bone marrow with a brief conceptual framework for structural and functional relationships among the different components of bone marrow. For a more thorough discussion of the biochemical and molecular control of hematopoiesis and the hematopoietic microenvironment, the reader is referred to Chapters 1 and 5.

SUPPORTING STRUCTURES

Hematopoietic tissue resides within a rigid boney cortex, and is further supported by a meshwork of trabecular bone that serves as a partial scaffold for additional structural components including adipose, reticular cells, and extracellular matrix. In addition to providing physical support, each of these structures contributes to the biochemical microenvironment of

hematopoietic tissue, either directly or via vascular connections.

A one to two cell thick layer of flat endosteal cells with a thin layer of connective tissue lines all of the boney surfaces within the medullary cavity. This layer is punctuated with occasional osteoblasts and osteoclasts, and may be traversed by endosteal blood vessels connecting the hematopoietic space with bone. Osteoblasts contribute to bone production and are derived from multipotent mesenchymal stromal progenitor cells that also give rise to bone marrow stromal cells and adipocytes.³⁰ Osteoclasts are multinucleated cells derived from fused monocyte-macrophage precursors under the influence of numerous signals, including those from osteoblasts.³⁰ Osteoblasts and osteoclasts remodel bone within the marrow space, influencing the endosteal environment and probably contributing to regulation of hematopoietic stem cell proliferation and trafficking.²⁵ Osteoblasts and osteoclasts also produce hematopoietic cytokines, and interplay between bone and hematopoietic cells can influence bone turnover and remodeling.^{30,36}

Fine, spindle to stellate stromal cells extend from endosteal regions into the parenchyma of hematopoietic tissue. These cells probably derive from fibroblasts in the bone marrow, and form a supporting meshwork for hematopoietic cells, adipose tissue, and blood vessels. Stromal cells produce soluble factors that contribute to regulation of hematopoiesis, and communicate with hematopoietic precursors via direct cell to cell contact. Bone marrow stromal cells produce structural fibrils such as collagen, reticulin, laminin, and fibronectin, and ground substance composed of water, salts,

glycosaminoglycans, and glycoproteins, which collectively are called the extracellular matrix. Like the supporting cellular structures of the marrow, the extracellular matrix participates in both the structural and biochemical support of hematopoiesis.²⁶

Bone marrow contains predominantly types I and III collagen, which take their final form after secretion into the extracellular space, where they undergo enzymatic modification. Reticulin is distinguished from collagen in the marrow by the presence of fine argyrophilic fibers, which are composed of a core of type I collagen surrounded by type III collagen fibrils embedded in a matrix of glycoproteins and glycosaminoglycans.²⁶ Although the fine connective tissue structure of bone marrow is not prominent on routinely processed histology sections, special stains can enhance its visualization. Collagen can be visualized with Mallory's or Masson's trichrome stains, whereas Gomori's silver stain highlights the presence of reticulin. This can be important in pathologic conditions in which production of excess matrix material contributes to disease. Differentiation of collagen and reticulin fibrosis may have diagnostic implications and influence the likelihood of reversibility of the lesion. Expanded extracellular matrix characterizes other bone marrow disorders beyond classical syndromes of myelofibrosis.^{26,41}

Adipose tissue interspersed with hematopoietic tissue is enmeshed in the same supporting structures. The relationship between formation of bone and adipose tissue is not clearly understood.¹⁵ Adipocytes probably are derived from the same mesenchymal progenitors that produce stromal cells and osteoblasts, and there is some evidence for interconversion of cells originating from committed osteoblastic and adipogenic lineages

derived from the mesenchymal progenitor population.³ Adipocytes are the most numerous stromal cells in bone marrow. In health, adipose tissue takes up approximately 25% to 75% of the bone marrow space, depending on the age of the animal. Adipose tissue also contains other cell types that are less visible than adipocytes on routine histologic sections. These cells also contribute to the structural and functional roles of adipose tissue in hematopoiesis, and include endothelial cells, macrophages, and adipocyte progenitor cells.²⁴ Both brown and white adipose tissue are present in bone marrow; differences in biological function of these types of fat are not fully understood. Bone marrow adipose tissue tends to be relatively resistant to lipolysis during starvation compared with adipose tissue elsewhere in the body.

In addition to providing structural support, adipose tissue also may participate in the hematopoietic microenvironment. Cells derived from bone marrow adipose tissue are capable of supporting differentiation of hematopoietic progenitors *in vitro*.^{8,10} The endocrine and paracrine functions of adipose tissue also are important.^{12,37} Adipokines, which are biologically active substances produced by adipose tissue, include regulators of hematopoiesis and the immune response.^{10,24,27,28,35}

VASCULATURE AND SINUS ARCHITECTURE

The nutrient artery provides the major blood supply to bone marrow (Fig. 2.1). Nutrient arteries enter the medullary cavity via one or more nutrient canals, which also may contain one or two nutrient veins. There often are two nutrient arteries for long bones, and flat bones may contain several.³⁹ Once the vessels have penetrated the

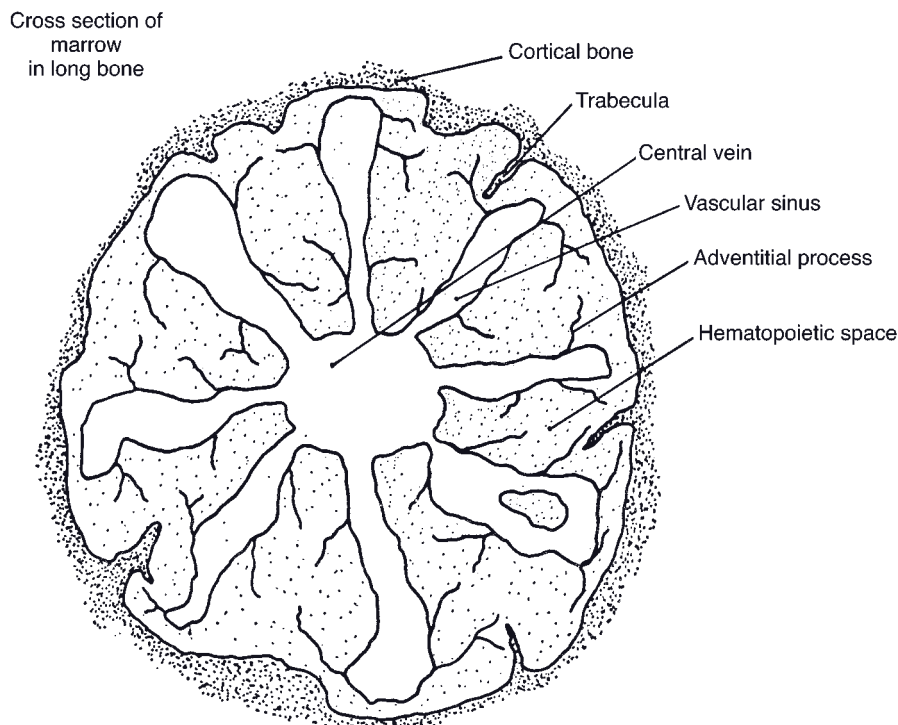


FIGURE 2.1 Organization of the venous vasculature of the marrow of a long bone. Thin-walled vascular sinuses originate at the periphery from termination of transverse branches of the nutrient artery (not shown). The vascular sinuses run transversely toward the center to join the CV. Hemopoiesis takes place in the space between the vascular sinuses. Adventitial processes project into the hematopoietic space, producing partial compartmentalization. (Reproduced from Lichtman MA. The ultrastructure of the hemopoietic environment of the marrow: a review. *Exp Hematol* 1981;9:391–410, with permission.)

cortex, ascending and descending branches bifurcate from the main vessels, coiling around the main venous bone marrow channel and central longitudinal vein. These branches form numerous arterioles and capillaries that penetrate the endosteal surface of the bone to communicate with cortical capillaries derived from arteries that supply surrounding muscle tissue (Fig. 2.1). These interactions facilitate communication and reciprocal regulation between hematopoietic cells and bone.³⁰ Capillaries derived from the nutrient artery extend as far as the Haversian canals before coursing back to the bone marrow and opening into the venous sinuses.

Periosteal arterioles penetrate cortical bone to form a second arterial system for the bone marrow. These vessels form branching networks of medullary venous sinuses. Medullary venous sinuses collect into the large central venous sinus from which blood enters the systemic circulation via the emissary vein, which exits through the nutrient foramen.¹

Hematopoiesis occurs in the extravascular spaces between the venous sinuses, and has a close morphologic and functional relationship with the cells that line the venous sinuses. Venous sinuses are lined by a complete luminal layer of broad, flat endothelial cells and an incomplete outer layer of abluminal reticular cells (Fig. 2.2). Reticular cells maintain close physical relationships with the hematopoietic cells close to the sinus walls, frequently wrapping around or otherwise contacting hematopoietic precursors. The basement lamina between the sinusoids and the hematopoietic space is thin and interrupted to facilitate release of mature hematopoietic cells into the circulation.²⁹ Sinus endothelial cells may regulate translocation of cells and other substances into the systemic circulation.

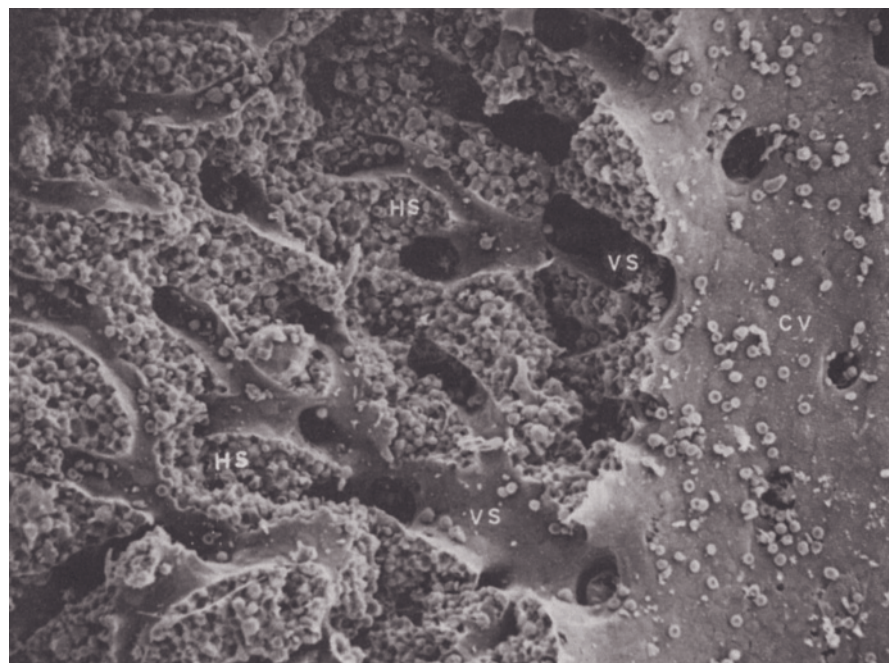
Ultrastructurally, sinus endothelial cells have distinct cell junctions that are not tight, and egress of hematopoietic cells through migration pores in endothelial cells has been observed. Other particulate matter may traverse sinus endothelial cells by the process of endocytosis.²⁹

INNERVATION

Primary innervation of the bone marrow is via myelinated and more numerous non-myelinated fibers. These fibers originate in the spinal nerve corresponding to the location of the nutrient foramen, although some innervation may originate from the epiphyseal and metaphyseal foramina.^{6,39} Once inside the medullary cavity, the mixed myelinated and non-myelinated nerve bundles, surrounded by a thin perineurium, divide to parallel the arterial vasculature of the bone marrow.⁶ The main branches of the arterial vessels are surrounded by several nerve bundles, whereas arterioles and capillaries may be accompanied by only a single fiber, with nerve endings contacting vascular smooth muscle cells or periarterial adventitial and reticular cells.⁴³

The sinusoidal system is less richly innervated than the arterial vasculature, with nerve endings frequently contacting the walls of sinusoids. Other nerve fibers appear to terminate within the hematopoietic parenchyma or along the endosteum.^{6,11} It is not clear if there is shared or separate innervation of bone and hematopoietic tissue, although at least some nerve fibers in the bone marrow appear to originate from mineralized bone.^{31,34} Bone marrow contains efferent noradrenergic and peptidergic sympathetic and presumptive sensory nerve fibers.^{9,34} There is some evidence that

FIGURE 2.2 Scanning electron micrograph of a rat bone marrow showing developing cells in hematopoietic spaces (HSs), anastomosing venous sinusoids (VS), and central vein (CV). 290 \times . (Prepared with assistance of Dr. Prem Handagama.)



signals from the sympathetic nervous system may contribute to regulation of hematopoiesis, immune function, and hematopoietic stem cell trafficking.^{2,5,9,20,22,32,38}

CELLULAR ORGANIZATION

An intricate three-dimensional complex of hematopoietic cells forms cords or wedges between vascular sinuses within the medullary cavity (Fig. 2.3). Different cell lineages occupy specific locations: granulocytes, lymphocytes, and macrophages are concentrated near the endosteum and arterioles, and megakaryocytes and erythroid cells are located near venous sinuses.^{1,14,33}

Hematopoietic cells are derived from a common pluripotent stem cell which gives rise to lymphoid and myeloid progenitor cells. Lymphoid progenitor cells generate lymphocyte progeny, whereas the myeloid progenitor cells generate erythroid cells, megakaryocytes, basophils, eosinophils, and a common granulocyte-macrophage cell that produces neutrophils and macrophages. Each cell line exhibits a pyramidal progression of cell numbers with the least mature cells

present in the smallest numbers and cells in subsequent stages of development present in increasing proportions.

Megakaryocytes

Megakaryocyte development begins with the megakaryoblast, progresses to promegakaryocytes and basophilic megakaryocytes, and culminates with formation of mature megakaryocytes. Megakaryocyte precursors progressively enlarge as they differentiate to become the largest cell in the bone marrow.^{4,16} Their nuclei mature from a single nucleus to a large multi-lobulated nucleus through a process termed endomitosis, which is replication of DNA without cellular division.^{4,16} The cytoplasm of early megakaryocytic precursors is scant and deeply basophilic, becoming more abundant, lightly basophilic, and filled with numerous eosinophilic granules as the cell matures.¹⁶

As megakaryocytes mature, they migrate toward the venous sinuses and may compose part of the endothelial cell layer.^{1,4,13,17,19,33,39,40} This location enables

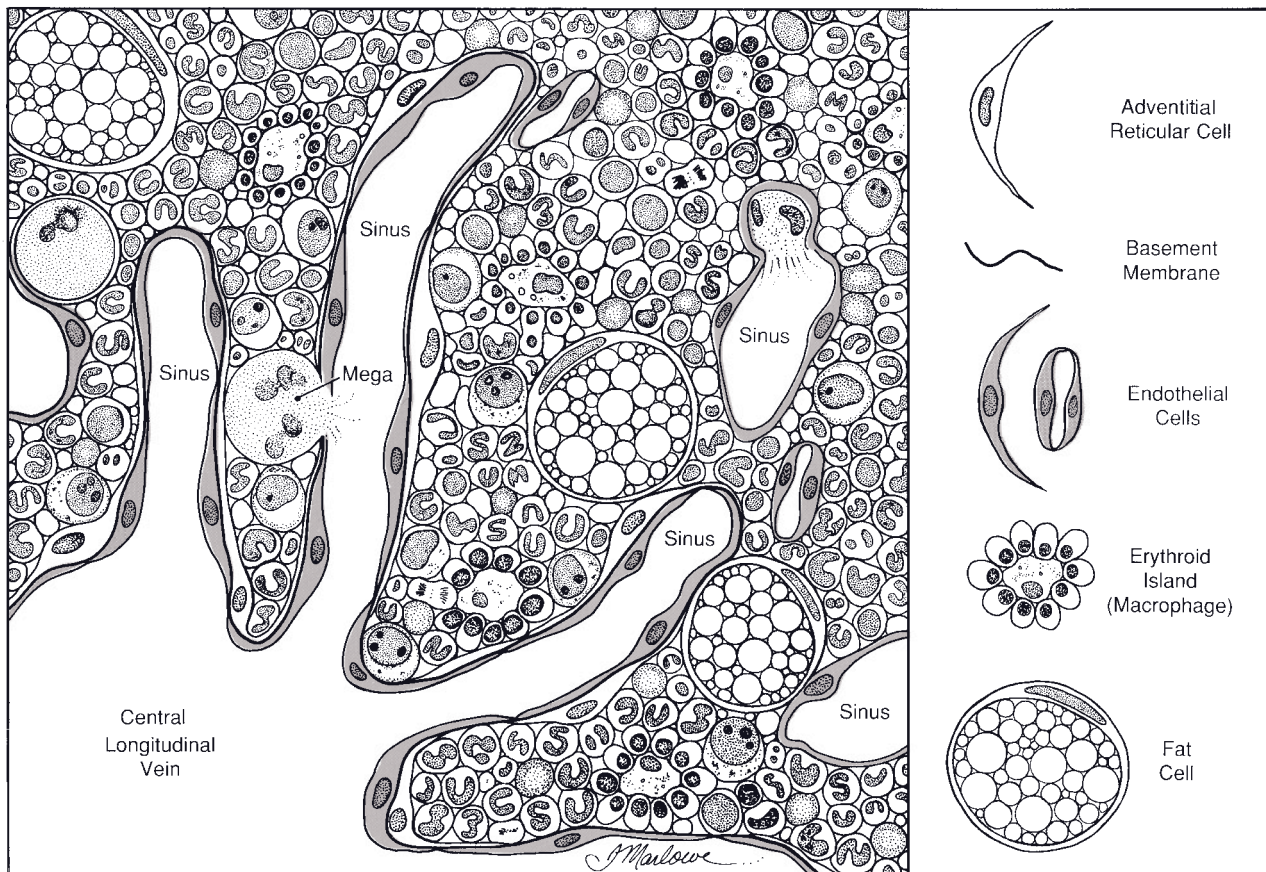


FIGURE 2.3 Schematic depiction of the hematopoietic compartment of the medullary cavity. Hematopoiesis occurs in cords composed of differentiating hematopoietic cells, stromal cells, adventitial reticular cells, adipocytes, and endothelial cells. Megakaryocytes (Mega) reside near the sinuses, shedding platelets directly into the sinus. Erythropoiesis occurs around macrophages, termed erythroid islands. Some evidence suggests structural variation in the location of primitive versus differentiated cells, with more primitive cells located nearer the bone surface. (Reproduced from Sieff C, Williams D. Hemopoiesis. In: Handin R, Lux S, Stossel T, eds. Blood: Principles and Practice of Hematology. Philadelphia: JB Lippincott, 1995, with permission.)

cytoplasmic processes to extend through endothelial gaps and discharge of proplatelets directly into the lumen of the sinus. Platelets are released from proplatelet processes into the peripheral circulation (see Chapter 9).^{13,17,19,39}

Erythroblastic (Rubriblastic) Islands

Stages of erythropoiesis include rubriblasts, prorubricytes, rubricytes, metarubricytes, reticulocytes, and mature RBCs (see Chapter 6).^{15,16,17} As erythroid precursors mature, the cells become smaller, the nuclear to cytoplasmic ratio decreases, the cytoplasm becomes less basophilic and more polychromatophilic, and the nuclear chromatin becomes condensed. In mammals, the nucleus is extruded before maturation to a mature RBC.^{16,17} Erythropoiesis occurs in distinct erythroblastic islands (Fig. 2.3), which are clusters of cells that occasionally can be observed in cytologic samples of bone marrow.^{1,7,39,44} Erythroblastic islands form around a central macrophage that projects membranous processes to assist erythropoiesis by providing iron and probably other nutrients and hematopoietic cytokines. These macrophages also phagocytize extruded nuclei and defective cells.^{1,7,13} Erythroid progeny are found in concentric circles surrounding the central macrophage with younger forms closer to the macrophage.¹ Central macrophages are recruited from a subset of resident macrophages derived from monocyte precursors. Erythroblastic islands are located near venous sinuses.^{1,39,40} A recent study of erythropoiesis in rat bone marrow suggests that erythroblastic islands are motile and migrate towards sinusoids as they mature, and that erythroblastic islands are composed of cells in similar stages of erythroid development.^{7,44}

Granulocytes

Neutrophils, eosinophils, and basophils develop in a parallel fashion from myeloblasts, promyelocytes, myelocytes, metamyelocytes, and band forms, to mature cells. Granulocytes, like RBCs, decrease their cellular size and nuclear to cytoplasmic ratio as they mature. Secondary specific granules appear in the myelocyte stage and allow differentiation of the various granulocytic lineages.^{16,17} At the metamyelocyte stage, the round nucleus elongates and indents to form a bean-shaped nucleus before ultimately forming segmentations at full maturation. The location of granulocytic cells is dependent on their stage of maturity. Immature forms are located near arterioles and boney trabeculae. As maturation proceeds, precursors migrate toward venous sinusoids, where they access the peripheral circulation by diapedesis.^{1,13,33,40}

Lymphoid Cells and Macrophages

Lymphoid progenitors produce B cells, which develop further in the bone marrow, and T/NK progenitors, which leave the bone marrow for further development

in the thymus and other tissues. Immature lymphoid cells and macrophages are located near the endosteum and arterioles,^{1,18,33} whereas mature lymphocytes are relatively uniformly distributed with the bone marrow parenchyma.^{1,18,33} Morphologic changes in lymphoid cells during maturation are relatively minimal compared to other lineages and include decreasing cell size, decreasing cytoplasmic basophilia, and increasing condensation of nuclear chromatin.

Stem Cell Niches

Hematopoietic stem cells (HSCs) are morphologically indistinct, and evaluation of surface proteins is required for definitive identification (see Chapter 3). HSCs reside in the bone marrow within stem cell niches, the specialized microenvironments created by supporting cells that provide the necessary signals for stem cell maintenance and function.^{21,23} Current evidence indicates that there are two niches: an endosteal or osteoblastic niche and a vascular niche.^{21,23,42} The endosteal niche is located adjacent to the endosteal surface of the bone marrow, in a location influenced by osteoblastic activity, and it promotes HSC quiescence and trans-marrow migration.^{21,23} The vascular niche is located near the sinusoidal endothelium and is involved in HSC expansion and egress into the circulation.²¹ The relationship between the two stem cell niches is still under investigation.^{23,42}

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Stem Cell Biology

JED A. OVERMANN, JAIME F. MODIANO, and TIMOTHY O. O'BRIEN

Defining Stem Cells

Characteristics

Tests and Markers

CD34

Stem cell antigen

Dye efflux

c-Kit

Lin⁻

Transcription factors

Bone Marrow-Derived Stem Cells

Stem Cell Biology

Regulation of Survival and Pluripotency

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Molecular mechanisms

Leukemia inhibitory factor

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Wnt

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Regulation of Differentiation

Stem Cell-Associated Diseases

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Stem Cells and Proliferative Disorders

Acronyms and Abbreviations

ABC transporter, ATP-binding cassette transporter; BMP, bone morphogenic protein; CD, cluster of differentiation; EPC, endothelial precursor cell; EPO, erythropoietin; ERK, extracellular signal related kinases; ESC, embryonic stem cell; FGF, fibroblast growth factor; GM-CSF, granulocyte/macrophage colony stimulating factor; HSC, hematopoietic stem cell; IGF-2, insulin-like growth factor 2; IL, interleukin; JAK/STAT, Janus kinase/signal transducers and activators of transcription; LIF, leukemia inhibitory factor; Lin⁻, lineage negative; miRNA, microribonucleic acid; MSC, mesenchymal stem cell; PE, phycoerythrin; PI3k, phosphoinositide-3 kinase; Sca-1, stem cell antigen 1; SCF, stem cell factor; SCID, severe combined immunodeficiency; SP, side population; Tie, tyrosine kinase with immunoglobulin-like and EGF-like domains 1; TNK, T cell and natural killer cell progenitor; TPO, thrombopoietin.

DEFINING STEM CELLS

Characteristics

Stem cells are a population of unspecialized precursor cells that have capacity for self-renewal and the ability to differentiate, leading to formation of mature cells and tissues. This latter function is clearly evident in embryonic stem cells (ESCs), as they lead to the establishment of the numerous different cells and tissues of the mature organism. Small numbers of stem cells are retained throughout life as adult stem cells and are a reservoir for replacement of short-lived cells or regeneration of damaged tissues. Hematopoietic stem cells (HSCs) are the reservoir for replacement of blood cells and are present in a frequency of 1 in every 10,000 to 100,000 blood cells.³ (Fig. 3.1; see Chapters 6–10).

Two general functional characteristics are used in defining stem cells. The first of these is the ability of long term self renewal. Stem cells have the capability, through mitotic cell division, to maintain a population of undifferentiated cells within the stem cell pool for months to years, and over many cycles of cell division. As stem cells divide, on average, one daughter cell is a replica and remains in an undifferentiated state, while the second daughter cell is programmed to differentiate. This production of two daughter cells with different properties is termed asymmetric cell division. The second characteristic of stem cells is the capacity to form differentiated or specialized cell types.

Potency is a term that is used to describe the degree or extent to which multiple functional cell lines can be formed. Totipotent stem cells are those cells that have the ability to form entire organisms, including extra-

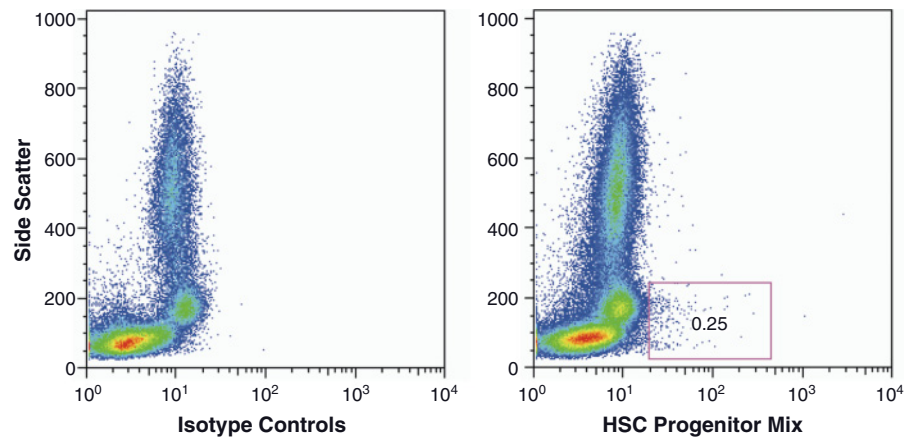


FIGURE 3.1 Hematopoietic progenitor cells in the peripheral circulation. Hematopoietic progenitor cells can be defined by expression of specific cell surface markers such as CD34, c-Kit, and CD133. In this case, such cells are detectable in blood from a normal dog using flow cytometry. Each panel shows a two-dimensional dot plot of FL2 (fluorescence channel-2 set to detect wavelength emission maxima at 575 ± 13 nm) vs. right angle side scatter. Cells were stained using routine protocols; dead cells were excluded using a vital dye. The left panel shows cells stained using an isotype control antibody. The right panel shows cells stained using a mix of antibodies against CD34, c-Kit, and CD133, each labeled with phycoerythrin (PE). In this healthy adult dog, approximately 0.25%, or $\sim 2/1,000$ viable leukocytes expressed one or more of the progenitor markers. While the frequency is almost 20-fold greater than that seen in most healthy dogs, this case serves to illustrate the presence of hematopoietic progenitor cells in circulation with no associated pathology. (Analysis and figure courtesy of Megan Duckett, Masonic Cancer Center, University of Minnesota.)

embryonic tissues (e.g. placenta). This type of stem cell can be derived from the zygote or early blastomere. Pluripotent stem cells are able to form all cell types of the body (e.g. ESCs). Multipotent stem cells generate all differentiated cells of a particular lineage (e.g. HSCs), and will be of particular interest to the topics in this text. Finally, unipotent stem cells give rise to only a single cell line (e.g. spermatogonial stem cells).¹³

Tests and Markers

Functional assays to demonstrate the pluripotent or multipotent nature of stem cells have been achieved through in vitro formation of embryoid bodies, in vivo generation of teratomas in mice with severe combined immunodeficiency (SCID) after grafting of ESCs, and, in the case of HSCs, repopulation of the hematopoietic system of lethally irradiated mice following transplantation of unpurified bone marrow derived cells.^{12,21,24} The ability to identify and isolate stem cells, however, relies largely on the use of a variety of markers such as surface molecules, transcription factors, and dye efflux. Numerous markers are available, and frequently are used in combination, to identify pluripotent cells and stem cells within certain types of tissue. While some markers and tests are used more universally to recognize stem cells, special attention will be paid to those used in identifying bone marrow-derived stem cells.

Cluster of Differentiation (CD)34

CD34 is a cell surface glycoprotein that has traditionally been used in identification and purification of HSCs

and progenitor cells.¹ This marker appears to be highly conserved among mammalian species. Experimental evidence suggests that CD34 may be involved in cell adhesion of hematopoietic cells to stromal cells in the bone marrow microenvironment.¹⁰ More recently, however, CD34-negative HSCs called side population (SP) cells were identified. SP cells are thought to be some of the most primitive HSCs because of their high proliferative potential and extreme efficiency at homing to sites of hematopoiesis when injected into recipient mice.¹⁹ CD34 expression on HSCs may thus be related to the degree of activation of these cells, with CD34-negative cells being the most primitive and quiescent.¹

Stem Cell Antigen

Stem cell antigen-1 (Sca-1) is a cell surface protein often used in identification of murine HSCs. This molecule may play a role in lineage determination.⁵

Dye Efflux

The ability of some primitive HSCs to efflux fluorescent dye allows for identification of this population, termed SP cells, by flow cytometry.^{8,19} This ability appears to be due to increased number or activity of membrane pumps (e.g. ATP-binding cassette transporter [ABC-transporter]), a hypothesis supported by the finding of blockage of dye efflux by the drug verapamil, a known inhibitor of these efflux pumps.⁸ SP cells lack CD34 expression and have been described in multiple species.⁹ As stated earlier, these CD34-negative cells have been proposed to be some of the most primitive HSCs.

c-Kit

c-Kit is a transmembrane tyrosine kinase receptor found on HSCs of multiple species. It binds the ligand stem cell factor (SCF, also called Steel factor), and is important in the maintenance, proliferation, and differentiation of HSCs.²⁸

Lin⁻

As an adjunct to the presence of certain markers (e.g. CD34, c-Kit, Sca-1), the absence of markers present on differentiated cells has been used to isolate and purify HSCs. A lineage negative (Lin⁻) classification generally indicates that cells are negative for a combination of anywhere from 6 to 14 different lineage markers of mature blood cells.

Transcription Factors

Transcription factors that appear to be important in regulation of stem cell pluripotency and their undifferentiated state have been identified. Most notable are the transcription factors Oct-4, Nanog, and Sox-2, which have been used as markers of embryonic and adult stem cells.⁶

BONE MARROW-DERIVED STEM CELLS

Within the bone marrow, there appear to be at least three different types of stem cell. HSCs are multipotential stem cells that give rise to the mature cellular elements of the blood (e.g. RBCs, neutrophils, monocytes, platelets, etc.). The stromal components of bone marrow such as bone, cartilage, fat, and fibrous connective tissue are derived from mesenchymal stem cells (MSCs), also termed marrow stromal cells. Finally, endothelial precursor cells (EPCs) are a population of bone marrow-derived cells that function in angiogenesis. EPCs are mobilized from the bone marrow into the peripheral blood, where they home to sites of neovascularization such as those present in areas of inflammation, tumor vascularization, or wound repair (see Chapter 11).¹

STEM CELL BIOLOGY

Regulation of Survival and Pluripotency

Niche

The niche concept is important to the discussion of stem cell survival and differentiation. Niches are local tissue microenvironments that function to support, maintain, and regulate stem cells. These microenvironments are found in various tissues throughout the body, for example the bulge region of the hair follicle, near the base of crypts in the gastrointestinal tract, and, in the case of HSCs, adjacent to endosteum and bone marrow

sinusoids.²⁰ Regulation of stem cells by their niche occurs through physical contact and cell-cell interactions with adjacent cells, as well as elaboration of soluble factors.²⁰ Evidence also indicates that stem cells have the ability to influence the cellular elements of their niche. For example, HSCs from mice subjected to an acute hematopoietic stress have an increased ability to direct bone marrow mesenchymal cells toward osteoblastic differentiation as a result of HSC-derived bone morphogenic proteins (BMPs).¹⁵

Molecular Mechanisms

What are the factors that cause stem cells to go down a pathway of self-renewal and remain undifferentiated versus progression toward lineage differentiation and mature cell phenotypes? The answer to this question is constantly evolving as specific molecular mechanisms are elucidated. Several factors important in the maintenance of stem cell survival, self-renewal, and pluripotency have been revealed in murine ESCs. These factors have been divided into extrinsic factors (e.g. cytokines) and intrinsic factors (e.g. transcription factors).

Leukemia Inhibitory Factor (LIF) LIF is an interleukin (IL) 6 class cytokine that prevents differentiation of mouse ESCs in culture. Binding of LIF to its membrane receptor results in activation of multiple molecular signaling pathways such as Janus kinase/signal transducers and activators of transcription (JAK/STAT), phosphoinositide-3 kinase (PI3K), and extracellular signal related kinases (ERK). Although these pathways are common downstream signals of many cytokines, in this context, their activation tends to promote maintenance of self-renewal and pluripotency. Activation of ERK in this example, however, appears to favor differentiation of mouse ESCs. Thus, LIF can activate signals that either promote or inhibit maintenance of an undifferentiated state, and it is the balance between these downstream effects (generally favoring self-renewal and pluripotency) that determines the outcome.⁶

Bone Morphogenic Protein 4 (BMP4) and Basic Fibroblast Growth Factor (FGF) BMP4 and basic FGF are additional examples of extrinsic factors that promote self-renewal and pluripotency in mouse ESCs. In the case of BMP4, it appears to work in a synergistic state with LIF.⁶ Discovery of additional signaling pathways and factors is likely, as factors important for mouse ESCs are not universal when applied to human ESCs.

Wnt Specific extrinsic factors involved in self-renewal of HSCs have been identified. The Wnt signaling pathway stimulates self-renewal of HSCs while concurrently inhibiting HSC differentiation. Inducing β -catenin activation, a downstream component of the Wnt signaling pathway, results in increased self-renewal of murine HSCs and limits differentiation of these cells. When inhibitors of the Wnt pathway are added to murine HSCs and growth factors, HSC proliferation is repressed.²²

Tyrosine Kinase with Immunoglobulin-Like and Endothelial Growth Factor-Like Domains 2 (Tie2) and Angiopoietin-1 Tie2/Angiopoietin-1 signaling has also been implicated in survival of HSCs. Tie2 is a receptor tyrosine kinase expressed on some HSCs. Angiopoietin-1 is the ligand for the Tie2 receptor and promotes quiescence and increased adhesion of murine HSCs to bone marrow stromal cells. Regulation of the quiescent state and maintenance within the HSC niche is thought to be important in HSC survival through a protective effect against myelosuppressive stresses.²

Other Cytokines Other cytokines important in regulation of HSC survival include SCF, thrombopoietin (TPO), BMP, FGF, insulin-like growth factor 2 (IGF-2), and interleukin (IL)-10. SCF and TPO are common components of most cytokine combinations used in the culture and propagation of HSCs. Although TPO is the primary cytokine involved in megakaryocyte and platelet production, it also has been shown to have significant effects on HSCs. In vitro, TPO promotes survival and expansion of HSCs, and mice that have been genetically altered to lack TPO or its receptor have significantly fewer stem cells.^{16,29}

Transcription Factors The intrinsic factors governing the undifferentiated state of ESCs consist primarily of transcription factors. Most notable are Oct-4, Nanog, and Sox-2. These factors are found in pluripotent cell lines and, in general, down-regulation results in differentiation of stem cells. Presence of Oct-4 and Sox-2 appears to be essential for pluripotency; however, the target genes for these transcription factors have not been completely characterized.⁶ Several transcription factors and cell cycle regulators governing self-renewal of HSCs also have been described.

Microribonucleic Acids (miRNAs) miRNAs are an additional intrinsic molecular mechanism proposed to be involved in maintenance of pluripotent stem cells. miRNAs are short, single-stranded RNA molecules that regulate gene function by suppression of translation through annealing and sometimes degradation of mRNA. Novel miRNAs have been found that appear to be expressed preferentially in undifferentiated ESCs. In addition, evaluation of miRNA expression profiles from ESCs of varying degrees of differentiation as well as cells from mature tissues show repression or loss of specific miRNAs as cells progress to a more differentiated state.¹¹

Regulation of Differentiation

Differentiation of stem cells into specific lineages is controlled or directed by factors including cytokines, niche interaction, and regulators of self-renewal and pluripotency. Cytokines influence and guide lineage determination of stem cells and many have been described in the context of HSCs and hematopoietic progenitor cell differentiation (Fig. 3.2; see Chapters 6–11).

Stromal cells that constitute the stem cell niche influence differentiation and lineage determination through

physical cell-cell interaction and elaboration of soluble or cell-bound factors (e.g. cytokines). Finally, as previously described, there are regulators that promote self-renewal and the pluripotent state of stem cells. For differentiation to occur, these regulators must be inhibited or suppressed. Mechanistically, there may be two general categories by which cells restrict lineage commitment.²³ The first of these involves the spectrum of surface receptors, adhesion proteins, and signaling pathways expressed by a given cell. For example, cytokines play an important role in lineage development. However, if a stem or progenitor cell lacks a cytokine receptor then that cytokine would have little or no effect on its target. Gene silencing is a second mechanism by which lineage restriction may occur. For cells to differentiate, specific genes are activated or silenced, guiding cells toward a particular lineage. This may be accomplished through mechanisms such as DNA methylation and histone modification, which alter the transcriptional state of the chromatin.

STEM CELL-ASSOCIATED DISEASES

Stem Cell Failure

The hematopoietic system offers a clear illustration of the effects of stem cell failure. HSCs are responsible for the constant replacement of all cellular components of blood, with HSC failure, cytopenias (e.g. anemia, leukopenia, thrombocytopenia) and their associated clinical manifestations (e.g. lethargy, infection, hemorrhage) ensue. HSC failure can be the result of a number of underlying pathologic processes, including toxic or drug-mediated damage, immune-mediated damage, infectious agents (e.g. parvovirus, feline leukemia virus, and *Ehrlichia* spp.), insufficient stimulation by cytokines and growth factors, and disruption of or damage to the stem cell niche (e.g. myelophthisis, ischemia, inflammation).^{4,7,25,26} (See Section II.)

Stem Cells and Proliferative Disorders

Just as adult stem cells are responsible for replacement of mature cells and tissues, there is strong evidence that cells with stem cell properties underlie the pathology of at least some types of cancer. The hypothesis of cancer stem cells is based on a few basic observations. The first of these is the observation of tumor heterogeneity. Many tumors comprise cells with different morphologies and phenotypes that in some cases loosely resemble the tissue of origin. This suggests a certain degree of differentiation within a population of tumor cells, leading to variability in structure and function. A more primitive precursor cell (i.e. cancer stem cell) could presumably give rise to the different phenotypes within a tumor. The second observation is that transplantation of a tumor required relatively large numbers of cancerous cells, an indication that only small numbers of cells in a given tumor have the ability to form a tumor. Cancer stem cells are present in small numbers within

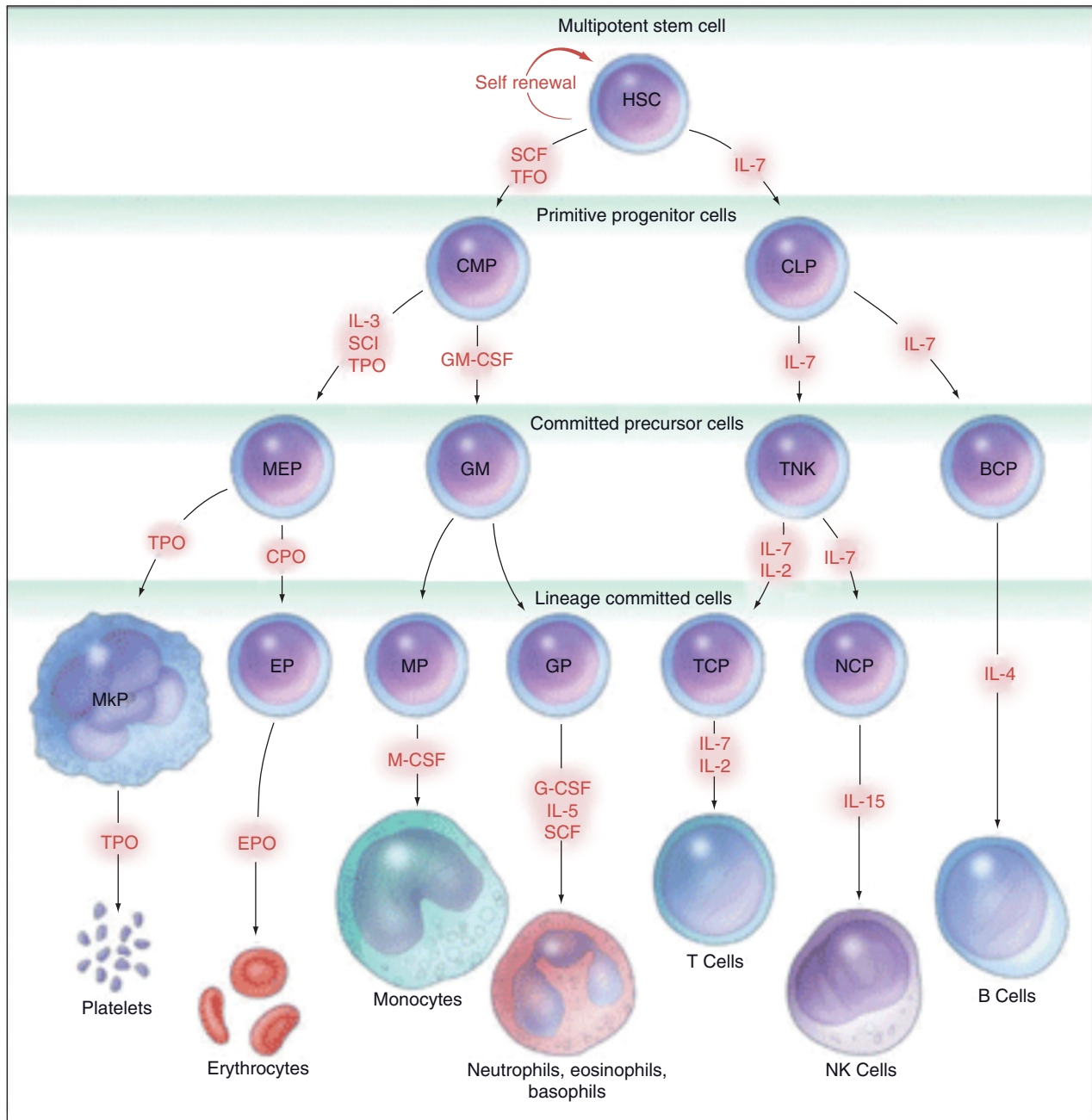


FIGURE 3.2 A general model of hematopoiesis. Blood cell development progresses from a hematopoietic stem cell (HSC), which can undergo either self-renewal or differentiation into a multilineage committed progenitor cell: a common lymphoid progenitor (CLP) or a common myeloid progenitor (CMP). These cells then give rise to more differentiated progenitors, comprising those committed to two lineages that include T cells and natural killer cells (TNKs), granulocytes and macrophages (GMs), and megakaryocytes and erythroid cells (MEPs). Ultimately, these cells give rise to unilineage committed progenitors for B cells (BCPs), NK cells (NKPs), T cells (TCPs), granulocytes (GPs), monocytes (MPs), erythrocytes (EPs), and megakaryocytes (MkPs). Cytokines and growth factors that support the survival, proliferation, or differentiation of each type of cell are shown in red. For simplicity, the three types of granulocyte progenitor cells are not shown; in reality, distinct progenitors of neutrophils, eosinophils, and basophils or mast cells exist and are supported by distinct transcription factors and cytokines (e.g. interleukin-5 in the case of eosinophils, stem-cell factor [SCF] in the case of basophils or mast cells, and G-CSF in the case of neutrophils). IL denotes interleukin, TPO thrombopoietin, M-CSF macrophage colony-stimulating factor, GM-CSF granulocyte-macrophage CSF, and EPO erythropoietin. (Reprinted from Kaushansky K. Lineage-specific hematopoietic growth factors. *New Engl J Med* 2006;354:2034–2045, with permission. ©Massachusetts Medical Society 2006.)

a tumor, and thus relatively large amounts of tissue would be needed to ensure the presence of these cells. Just like normal stem cells, cancer stem cells share the basic functional properties of self-renewal and the ability to differentiate.

In humans, evidence for cancer stem cells has been shown in hematopoietic, brain, breast, colon, prostate, bone, and ovarian cancers, and there is some evidence for the existence of cancer stem cells in animals.^{14,17,18,27} Support for the existence of cancer stem cells in humans consists of identification of a subset of tumor cells that express stem cell markers and have exclusive or enhanced ability to form tumors *in vitro* or *in vivo*. Evidence exists that cancer stem cells may arise from normal stem cells and/or progenitor cells that have reacquired the ability of self-renewal. The origins of cancer stem cells continue to be explored.

The existence of cancer stem cells has clear implications for understanding cancer biology and treatment in at least certain types of cancers. For example, many chemotherapeutics target rapidly dividing cells. However, cancer stem cells are relatively slowly cycling, thus allowing them to persist with these conventional treatments. Newer therapeutic modalities directed at elimination of cancer stem cells will be important for effective treatment of these types of neoplasia.

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Cluster of Differentiation (CD) Antigens

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Definition and History

Structure and Integration of Membrane Antigens

Transmembrane Proteins

Single Pass Type I Transmembrane Protein (I)

Single Pass Type II Transmembrane Protein (II)

Multipass Transmembrane Protein (III)

Glycosyl Phosphatidylinositol Transmembrane

Protein (V)

Tissue Distribution

Acronyms and Abbreviations

CD, cluster differentiation; CLAW, Canine Leukocyte Antigen Workshop; ER, endoplasmic reticulum; FIV, feline immunodeficiency virus; GP, glycoprotein; GPI, glycolyl phosphatidylinositol transmembrane protein; Ig, immunoglobulin; IL, interleukin; Mab, monoclonal antibody; MDR-1, multidrug resistance transporter protein; MHC, major histocompatibility antigen; NK, natural killer; PROW, The International Protein Reviews on the Web; VLA, very late antigen.

DEFINITION AND HISTORY

Cluster differentiation (CD) nomenclature was first introduced at an international conference in Paris (1982) during the boom in monoclonal antibody (MAB) technology.² This nomenclature was established to standardize the classification of cell surface antigens and to define the biological functions of molecules expressed by various hematopoietic lineages. Several international workshops have been held to exchange human and other species-specific MABs and to compare their reactivity with cells and cell proteins from veterinary species.^{7,27,28,40,46} Based on these workshops, MABs that have similar reactivity with tissues or cell types are assigned to a cluster group. Therefore, an antigen that is recognized by a cluster of MABs is assigned a “cluster of differentiation” (CD) number. If MABs defining a cluster of antigens are derived from the same laboratory, the suffix “w” is appended to the CD designation. Only eight CD antigens are internationally accepted as defined by the 1st Canine Leukocyte Antigen Workshop (CLAW) and include the following homologues to the human system: CD4, CD5, CD8, CD11a/18, CDw41, CD44, CD45, and CD45R.⁷ The final meeting of the Human Leukocyte Differentiation Antigen 8-workshop was held in December, 2004, in Adelaide, Australia, and 376 MABs from various companies, mainly directed against human leukocytes, were tested for their reactivity with cells from 17 different animal species. A special

issue of the journal *Veterinary Immunology and Immunopathology* described these efforts.³⁹

Today there are over 339 CD molecules defined. This explosion in CD molecules is the result of the use of molecular biology techniques to identify new molecules. The purpose of this chapter is to provide the most current listing of CD antigens recognized by various antibodies in veterinary species. Table 4.1 summarizes the current knowledge of important veterinary CD antigens, including MAB clones that react with dog, cat, ruminant (cattle, sheep, and goats), pig, and horse CD antigens; relative molecular size(s); topology in the membrane; tissue distribution; known physiology; and species reactivity of MABs. Selected key references for each CD antigen are included.

More in depth information is available for the currently described CD molecules on several websites, including the taxonomic key program maintained by Washington State University that is designed to provide information of the specificity of MABs specific for leukocyte differentiation molecules intra- and cross-species (<http://www.vetmed.wsu.edu/tkp/>). There are links from this website to commercial companies that are the major suppliers of veterinary-specific MABs, including Serotec (http://www.ab-direct.com/antibodies/_-500.html) and Veterinary Medical Research & Development (VMRD) (<http://www.vmr.com/>). The International Protein Reviews on the Web (PROW) (<http://mpr.nci.nih.gov/prow/>) provides an

TABLE 4.1 Cluster Differentiation Antigens

CD Antigen (MAb Clone)	MAb reactivity (primary species in <i>italic</i>)	Size (M _r)	Topology	Distribution	Physiology	Selected References
CD1a (CA9.AG5)	<i>Canine</i>	49	I	Thymocytes dendritic cells, Langerhans cells	Nonpolymorphic CD1 family of glycoproteins includes CD1a, b, c, d, and e isoforms involved in presentation of foreign and self lipid antigens and glycolipids; 43–49 subunits interact with a 12 kDa subunit (α_2 -microglobulin)	35, 26
CD1a8.2 (CA13.9H11)	<i>Canine</i>	43				26
CD1a6 (Fe1.5F4)	<i>Feline</i>	49				26, 53
CD1a (Fe15.5C1)	<i>Feline</i>					
CD1b (CC20)	<i>Bovine</i>	45				19
CD2 (MCA833F) (HB88A) ^b	<i>Bovine</i> <i>Equine</i>	45–58	I	T cells, NK cells	Enhances adhesion between T cells and antigen presenting cells	13, 23
CD3 (CA17.2A12) (MM1A) ^b	<i>Canine</i> <i>Bovine</i>	12, 16, 22, 32, 44	I	T cells	A family of proteins that forms a signal transduction complex for T cell receptor when it binds antigen)	11, 50
CD4 (CA13.1E4) (vpg34)	<i>Canine</i> <i>Feline</i>	60 55	I	Neutrophil, T cells T cells	Receptor for MHC class II, facilitates recognition of peptide antigens	7, 34 51
CD5 (YKIX322.3), (DH13A) ^b (HB19A), (HT23A) ^b	<i>Canine</i> <i>Equine</i>	67	I	T cells	Receptor for CD72, facilitates signals transduced by T cell receptor for antigen	3, 7 21, 23
CD8 alpha (CA9.JD3) (Fe1.10E9) (73/6.9.1) ^b	<i>Canine</i> <i>Feline</i> <i>Equine</i>	32, 36	I	Thymocytes, T cells	Forms a heterodimer with CD8 β ; receptor for MHC class I; facilitates recognition of peptide antigens	34 53 49
CD8 beta (CA15.4G2) (vpg9) (HT14A) ^b	<i>Canine</i> <i>Feline</i> <i>Equine</i>	39	I	T cells	Forms a heterodimer with CD8 α (see above)	34 44 49
CD9 (vpg15) (MM2/57) (RHIA) ^b	<i>Feline</i> Human, <i>canine</i> , <i>feline</i> , <i>bovine</i> , <i>horse</i> <i>Bovine</i>	24 30–45	III	Activated lymphocytes, monocytes, lymphocytes, granulocytes, platelets	Important for signal transduction, cell activation, adhesion, aggregation; co-receptor for FIV; associates with tetraspan superfamily (CD63, CD81, CD82)	18, 52 39, 41 47
CD11a (Ca11.4D3, Ca11.7H11)	<i>Canine</i> , <i>feline</i>	180, 95	I	Monocytes, granulocytes, lymphocytes	Associates with CD18 to form a heterodimer receptor to facilitate adhesion	9
CD11b (CCA16.3E10)	<i>Canine</i> , ruminants, porcine, <i>feline</i> , mink, human	180	I	Granulocytes, monocytes, lymphocyte subset	Associates with CD18 to form a heterodimer receptor to facilitate adhesion	3, 9, 10
CD11c (Ca11.6A1, Ca11.7D1)	<i>Canine</i>	150, 95	I	Dendritic cells, granulocytes, macrophages, lymphocytes	Associates with CD18 to form a heterodimer receptor to facilitate adhesion	9
CD11d (Ca11.8H2)	<i>Canine</i>	155	I	CD8+ T cells, $\gamma\delta$ - T cells, macrophages in splenic red pulp	Associates with CD18 to form a heterodimer receptor to facilitate adhesion	10

TABLE 4.1 *Continued*

CD Antigen (MAb Clone)	MAB reactivity (primary species in <i>italic</i>)	Size (M _r)	Topology	Distribution	Physiology	Selected References
CD13 (CVS19)	<i>Equine</i>	150	II	Granulocytes and monocytes	Aminopeptidase N, a metallopeptidase in humans which removes NH ₂ terminal amino acids from peptides	22, 23
(CC81)	<i>Bovine</i>			Dendritic cells in afferent lymph		17, 20
CD14 (CAM36) ^b	<i>Ruminant, canine, feline, porcine, llama</i>	53	V	Monocytes, myeloid cells	Receptor for lipopolysaccharide that transduces signals, leading to oxidative burst and proinflammatory cytokine synthesis	3, 31
CD18 (CA1.E49)	<i>Canine</i>	95	I	All leukocytes	Beta subunit of the integrin heterodimer and combines with the alpha subunit of either CD11a, CD11b, CD11c, CD11d; plays a role in adhesion to endothelium	33
(H20A) ^b	<i>Bovine</i> <i>Equine</i>					21 41
CD21 (CA2.1D6)	<i>Canine</i> <i>Equine, feline, human</i>	145–160	I	B-cells, monocytes follicular dendritic cells	Receptor for C3d fragment and CD23; enhances B cell antigen receptor signal transduction	3, 4 3, 4
(LB21)	<i>Feline</i>					31
CD22 (RFB-4) (Mc64-12)	<i>Human</i> <i>Canine</i> <i>Bovine</i>	130, 140	I	Early B-cell stages, mature B-cells	Binds sialoglyco-conjugates on some CD45 isoforms to modulate B cell signal transduction	16, 42, 47
CD23 (M763) ^a	<i>Human</i> <i>Ruminants, canine, feline, porcine</i>	44 52	II	B-cells and monocytes	Involved in the regulation of IgE synthesis following binding to IgE Fc and IgE-containing immune complexes	3
CD25 (CACT116A, CACT108A) ^b (9F23)	<i>Ruminants</i> <i>Feline</i>	50–55	I	Mitogen stimulated T or B lymphocytes, LPS stimulated monocytes	IL2-receptor-alpha subunit	36–38
CD29 (12G10, 3S3)	<i>Human,</i> <i>canine</i>	110	I	Platelets	Beta chain of VLA, platelet GPIIa	42
CD34 (1H6)	<i>Canine</i>	110	I	Lymphohematopoietic stem cells and progenitors, endothelial cells	Leukocyte-endothelial interactions through binding with CD62L and CD62E	29, 30
CD35 (To5) ^a	<i>Feline</i>	190, 220	I	B-cells, erythrocytes, granulocytes, monocytes	Receptor for C3b and C4b (CR1) bound to immune complexes	31
CD41 (Canine 20-4), (CL2A) ^b , (M7057) ^a	<i>Canine</i>	125	I	Platelets, megakaryocytes	Integrin αIIb CD41/CD51 complex, receptor for fibrinogen	3, 7
CD44 (YKIX337.8) (BAG40A) ^b	<i>Canine</i> <i>Feline, bovine</i>	90	I	Most cell types including epithelial cells, activated T cells	Receptor for hyaluronate that facilitates lymphocytic binding to high endothelial venules	7 7, 47
CD45 (CA12.10C12)	<i>Canine</i>	180, 200, 220	I	Pan leukocyte	Membrane-bound tyrosine phosphatase critical for antigen-receptor-mediated activation of leukocytes	4, 6, 7, 50
CD45RA (CA4.1D3)	<i>Canine</i>	205, 220, 180– 240	I	Naïve T/B lymphocytes	Largest of the CD45 isoforms	4, 6, 7, 50

TABLE 4.1 *Continued*

CD Antigen (MAb Clone)	MAb reactivity (primary species in <i>italics</i>)	Size (M _r)	Topology	Distribution	Physiology	Selected References
CD47 (HUH69A), (HUH71A) ^b	<i>Human</i> Canine Bovine	47–55	III	Lymphocytes, macrophages, granulocytes	Associates with CD61 integrins to form receptor for thrombospondin; role in chemotaxis and adhesive interactions with leukocytes and endothelial cells	39 42 47
CD49d (Fe 2.9F2), (P4G9) ^a	<i>Feline</i> , canine, bovine	180	I	Lymphocytes, macrophages, granulocytes	VLA binds with 39CD29; binds fibronectin and mucosal addressin	3, 31
CD49e (JBS5)	<i>Human</i> , canine	155	I	Macrophages, granulocytes	VLA antigen binds to CD29 and together is the fibronectin receptor and binds to RGD sequence of fibronectin	42
CD56 (MOC-1), (T199) ^a	<i>Human</i> , canine	175–220	I or V	Subset of lymphocytes	Homotypic adhesion and natural killer cell cytotoxicity	42
CD61 (Y2/51) ^a	<i>Human</i> , canine	90	I	Platelets, megakaryocytes, monocytes	Associates with CD41 to form the GPIIb-IIIa heterodimer that facilitates platelet aggregation	42
CD79a (HM57) ^a	<i>Human</i> , canine	44–49	I	B lymphocytes	Cytoplasmic molecule that mediates slg expression and B cell receptor cell transduction	16, 32
CD88 (S5/1)	<i>Human</i> , bovine	30–45	III	Monocytes, B cells, T-cell subset	C5a receptor homolog; G-coupled receptor that triggers chemotaxis, respiratory burst and degranulation of granulocytes in humans	45
CD90 (CA1.4G8), (CA9.GA11)	<i>Canine</i> , equine Porcine, mink Guinea pig	18–44 24 or 26 27	V	Pro-thymocytes, T-cells, monocytes; weak on granulocytes, renal tubular cells	May contribute to formation of neuron memory and to growth regulation of hematopoietic stem cells	3, 4, 7
CD91 (A2MR-2) ^b	<i>Human</i> , canine	600	I	Macrophages of liver, lung and lymphoid tissues	Member of low density lipoprotein receptor family that binds to α_2 macroglobulin	39, 42
CD94 (HP-3D9) ^b	<i>Human</i> , canine	30	II	Lymphocyte subset (NK)	Binds to NKG2 and plays a role in recognition of MHC class I molecules by NK cells and some cytotoxic T cells; ligation of CD94 can inhibit or stimulate killing by NK cells	42
CDw119 (BB1E2)	<i>Bovine</i>	90–100	I	Monocytes, B cells, epithelial cells	Interferon gamma receptor	47
CD134 (7D6)	<i>Feline</i>	43	I	CD4+ activated T cells	Tumor necrosis factor receptor superfamily; regulator of T cell-dependent immune responses; receptor for FIV in conjunction with CXCR4	14, 43
CD163 (Ber MAC3)	<i>Bovine</i>	130	I	Monocytes	Scavenger receptor cysteine rich family	47
CD172a (DH59b) ^b	<i>Bovine</i> , canine, equine, feline	90	I	Monocytes, granulocytes	Member of the signal regulatory protein family involved in negative regulation of receptor tyrosine kinase-coupled signaling processes	12, 15, 31, 39, 42
CD235a (JC159) ^a	<i>Human</i> Canine	35 20	I	Erythrocytes	Glycophorin A, major sialoglycoprotein on erythrocytes	42

Column 1: CD designation of the antigen with the monoclonal antibody (MAb) clone published to have primary reactivity shown below in parentheses. Column 2: primary species of reactivity (*italics*) and those species with cross reactivity (all antibodies are commercially available; those with superscripts are obtainable from Serotec, Inc.^a and VMRD^b). Column 3: relative molecular mass of the reduced CD antigen. Column 4: integration of antigen to plasma membrane. Column 5: cell types and tissues known to express a CD. Column 6: proposed or known physiology of a CD antigen based on studies in animals or humans. Column 7: key references concerning identification and characterization of the CD antigen and/or characterization of the antibodies.

exhaustive database of the complete listing of CD antigens and links to primary nucleic acid and protein sequences of human CD antigens.

STRUCTURE AND INTEGRATION OF MEMBRANE ANTIGENS

CD antigens are principally membrane proteins defined by their location within or at the surface of the phospholipid bilayer. Membrane proteins are classified into three categories: integral, lipid-anchored, and peripheral, depending on the nature of membrane-protein interactions.²⁴ The CD antigens are grouped as integral membrane proteins or transmembrane proteins and consist of cytosolic, membrane spanning, and exoplasmic (luminal) domains. The cytosolic and exoplasmic domains have hydrophilic exterior surfaces with either C-terminus or N-terminus group endings. The membrane-spanning domains usually contain hydrophobic amino acids and consist of one or more alpha helix or multiple beta strands.²⁴ Most integral membrane proteins fall into one of five classes, depending on how they anchor themselves in the membrane.¹ Type I and II proteins have a single transmembrane region, whereas Types III and IV have multiple transmembrane regions also referred to as tetraspanins. Type IV proteins (not shown in the table) are distinguished from type III proteins by the presence of a water-filled transmembrane channel. Type V proteins use lipid to attach to membranes. These lipids are either a glycosyl-phosphatidylinositol (GPI) anchor or lipid moieties such as myristoyl groups, which include cytoplasmic signaling proteins that will not be described in this chapter.^{1,25} Each class pertinent to CD antigens used in veterinary medicine will be described briefly, and examples of common CD antigens used in diagnostic veterinary medicine will be highlighted in the text and are listed in Table 4.1.

TRANSMEMBRANE PROTEINS

Single Pass Type I Transmembrane Protein (I)

Type I and II transmembrane proteins have only one membrane-spanning α -helix containing 20–25 hydrophobic amino acids. Type I proteins have an N-terminal endoplasmic reticulum (ER) signal sequence that is cleaved after the molecule passes into the ER. This is the most common mode of membrane integration among the CD antigens listed in Table 4.1. The protein is glycosylated in the Golgi apparatus if the protein has a glycosylation site and then is expressed on the cell surface. Type I proteins are anchored in the membrane with their hydrophilic N-terminal region on the exoplasmic face and their hydrophilic C-terminal region on the cytoplasmic face. These proteins commonly represent cell surface receptors and/or ligands (like CD4), of which many belong to the immunoglobulin (Ig) superfamily.¹

Single Pass Type II Transmembrane Protein (II)

Type II transmembrane proteins lack a cleavable ER signal sequence and are oriented with their hydrophilic C-terminus on the exoplasmic face and the hydrophilic N-terminus on the cytoplasmic face. These proteins have an internal hydrophobic ER signal and membrane anchor sequence. Because these proteins can be released from the cell surface, they also can act as plasma proteins with physiologic effect(s) on cells bearing the counter ligands.¹ CD13, a zinc-binding metalloprotease that acts to facilitate antigen presentation by trimming the N-terminal amino acids from MHC Class II-bound peptides, is an example of a type II protein.¹

Multipass Transmembrane Protein (III)

Type III transmembrane proteins cross the membrane multiple (2, 3, 4, 5, 7, or 12) times and also are called tetraspanins. The most frequently found tetraspanins include those that span the membrane four and seven times. Structural studies indicate that the transmembrane regions are alpha helices. If the type III protein has an even number of transmembrane alpha helices, its N- and C-termini are oriented toward the same side of the membrane. Many of these cell surface molecules function as receptors for soluble molecules such as prostaglandins and chemokines. Examples of seven transmembrane type III proteins are interleukin-8 (IL-8) receptor (CD128) and C5a receptor (CD88). CD9 is an example of a four multi-pass transmembrane protein.¹ CD47 is an example of type III protein with five transmembrane sequences. The multidrug resistance transporter protein MDR-1 (CD243) has 12 transmembrane regions.¹

Gpi Transmembrane Protein (V)

Type V transmembrane proteins utilize GPI anchors attached to the C-terminal residue of the protein. GPI-anchored molecules have a secretion signal sequence at their N-terminus and C-terminus that is cleaved and replaced by the GPI-anchor after synthesis of the molecule and entry into the ER.²⁵ CD14, a surface protein involved in the clearance of Gram-negative pathogens bound to lipid polysaccharide binding protein (Fig. 4.1), is an example of a GPI-linked glycoprotein. Other examples of GPI-linked glycoproteins include CD56 and CD90.¹

TISSUE DISTRIBUTION

Cell surface antigens commonly used in immunophenotyping of hematologic neoplasia initially were determined by studies of hematopoietic cell differentiation and maturation.^{8,50} Lineage-associated markers can be broadly classified into groups that recognize B cell, T cell, natural killer (NK) cell, myeloid/monocytic, and erythroid lineages. Noncommitted hematopoietic stem cells express CD34, a glycosylated surface glycoprotein.

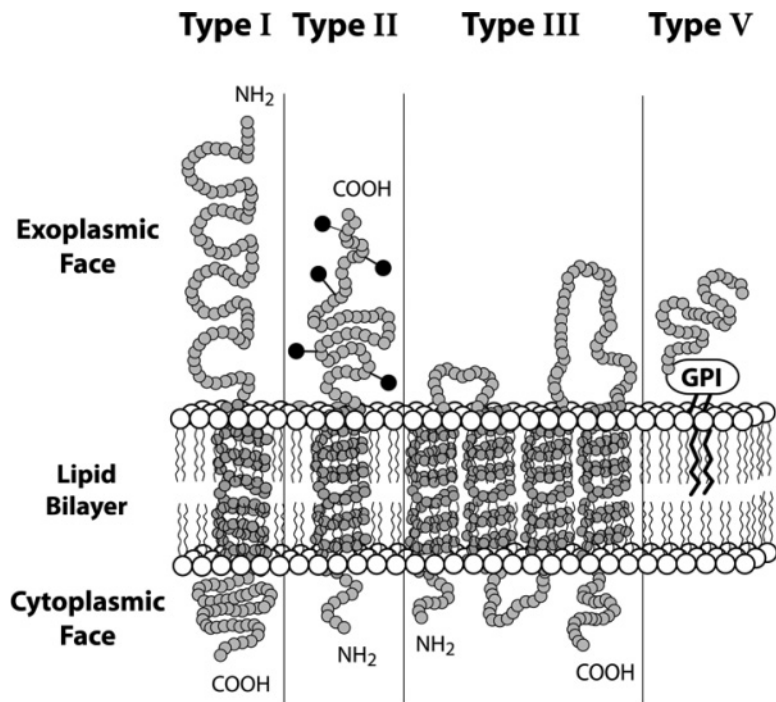


FIGURE 4.1 Major integral membrane proteins and their topology or interaction in the lipid bilayer. The types of membrane proteins are indicated at the top. CD4 is a type I transmembrane protein that passes through the membrane once, has four extracellular immunoglobulin domains, a carboxyl terminus on the cytoplasmic face of the bilayer, and N-terminus (NH₂) on the exoplasmic face. CD13 is a type II transmembrane protein with the N-terminus on the cytoplasmic face and the carboxyl terminus on the exoplasmic face. The extracellular domain of CD13 is heavily N-glycosylated (thin pegs extending from the polypeptide backbone). CD9 is a type III multi-pass membrane protein with four transmembrane regions, and N- and C-termini on the cytoplasmic face. CD14 is a type V glycosylphosphatidylinositol (GPI) anchored protein. (Courtesy of Mal Rooks Hoover, Graphic Design Specialist.)

This marker is frequently used to differentiate acute immature leukemias of lymphoid or myeloid origin from chronic lymphocytic leukemia or leukemic stages of lymphoma.¹ Lineage-specific cell surface antigens that are useful for delineating leukocyte cell lineages include CD3, which is exclusively expressed on mature T cells, and CD79 α and β , which form part of the B cell surface antigen receptor. Non-lineage restriction surface antigens include antigens such as CD45, which is found on all leukocyte lineages (myeloid and lymphoid). Although the last CLAW was in 1993, it was an important collaborative effort because anomalous expression of the CD4 antigen was identified in dogs, which express a high density of CD4 on neutrophils in addition to helper T cells.³⁴ Many other differences in CD antigen expression are unique among domestic animals compared to humans (e.g. high expression of the $\gamma\delta$ T cell receptor in pigs and ruminants^{5,36} and co-expression of CD4 and CD8 on mature T cells in swine).⁴⁸

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The Hematopoietic System

BRUCE D. CAR

Hematopoietic Stem Cells
 Hematopoietic Progenitors and Precursors
 Species Specificity of Hematopoiesis
 The Bone Marrow Microenvironment
 The Hematopoietic Stem Cell Niche
 The Erythropoietic Niche
 Cytokines and Cytokine Signaling in Hematopoiesis
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 Transduction

Evaluation of Hematopoietic Function
 Bone Marrow Evaluation in Mice
 Evaluation of Hematopoiesis with Bone Marrow
 Culture
 Lethal Irradiation and Bone Marrow Transplantation
 Genetically Altered Mice
 Models of Accelerated Hematopoiesis

Acronyms and Abbreviations

Ang1, angiopoietin 1; BFU-E, burst-forming unit erythroid; CD, cluster of differentiation; CFC-S, splenic colony-forming cell; CFU-E, colony forming unit erythroid; CFU-GEMM, colony forming unit granulocyte erythroid monocyte megakaryocyte; CFU-GM, granulocyte-macrophage colony forming cell; CLP, common lymphoid progenitor; CXCR4, CXC chemokine receptor 4; ECM, extracellular matrix; EDTA, ethylenediaminetetraacetic acid; EPO, erythropoietin; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte macrophage colony stimulating factor; GTPase, guanosine triphosphatase; HSC, hematopoietic stem cell; ICAM-4, intercellular adhesion molecule-4; IL, interleukin; JAK, Janus tyrosine kinase; JH, Janus homology; NK cell, natural killer cell; PCR, polymerase chain reaction; PI3K, phosphoinositol 3 kinase; PTH, parathyroid hormone; qPCR, quantitative polymerase chain reaction; Rac1 and Rac2, Ras-related C3 botulinum toxin substrate 1 and 2; RBC, red blood cell; SCF, stem cell factor; SCID, severe combined immunodeficiency; SDF-1, stromal derived factor 1 (CXCL12); SH, src homology; SOCS, suppressor of cytokine signaling; STAT, signal transducers and activators of transcription; TGF- β , transforming growth factor beta; Tie2, tyrosine kinase with immunoglobulin-like and EGF-like domains 2; TNF α , tumor necrosis factor alpha; TPO, thrombopoietin; TRAIL, TNF-related apoptosis inducing ligand; VCAM-1, vascular cellular adhesion molecule 1; VLA-4, very late antigen 4.

Current understanding of the hematopoietic system draws heavily from clinical observations and research in humans and mice. Numerous gene-deleted mice targeting transcription factors, hematopoietic cytokines and their receptors, and extracellular matrix (ECM) components and their receptors are central to our knowledge of hematopoiesis at the molecular level.⁷ More recent studies have contributed information about the critical role of the bone marrow microenvironment. In domestic animals, research on spontaneous hematopoietic neoplasia, the roles of retroviruses and viral oncogenes that mimic hematopoietic tyrosine kinases, cyclic neutropenia of gray collies,

and other species-specific hematopoietic diseases, has added to our understanding of hematopoiesis, which is remarkably conserved across mammalian species.³²

HEMATOPOIETIC STEM CELLS

Hematopoietic stem cells (HSCs), which are considered to have lost their potential for mesenchymal differentiation, arise first in the embryonic yolk sac, then in fetal para-aortic splanchnopleura, from which liver and finally bone marrow are seeded (see Chapter 1). In the conventional view of hematopoiesis, pluripotential

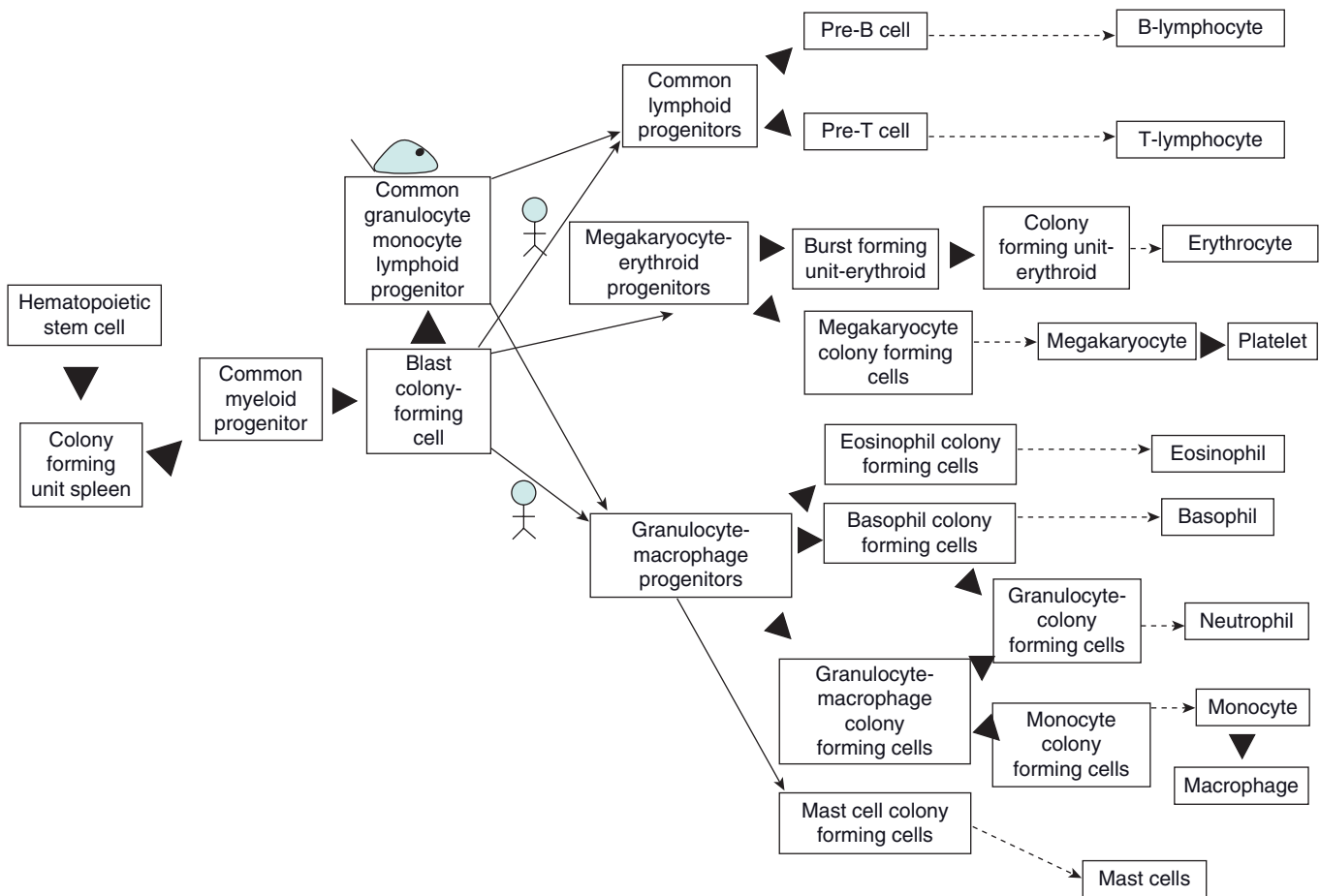


FIGURE 5.1 Hierarchical scheme of hematopoiesis. This figure depicts the conventional view of human and murine hematopoiesis. Larger block arrowheads indicate differentiation from pluripotent to oligopotent hematopoietic stem cells. Smaller block arrowheads indicate lineage commitment of oligopotent stem cells driven by cytokines and the JAK-STAT pathway. Dotted line arrows illustrate the multiple stages of differentiation that can be visually discriminated in Wright-Giemsa-stained bone marrow specimens. The figure has been modified from human and murine illustrations to indicate the mouse-specific GM-lymphoid precursors. (Modified from Iwasaki H, Akashi K. Hematopoietic development pathways: on cellular basis. *Oncogene* 2007;26:6687–6696 and Metcalf D. Hematopoietic stem cells and tissue stem cells: current concepts and unanswered questions. *Stem Cells* 2007;25:2390–2395.)

HSCs with unlimited self-generative capacity progressively differentiate to multipotent or oligopotent stem cells with reduced self-replicative capacity, to lineage committed progenitors with minimal ability to self-renew, to lineage-specific precursors with no self-regenerative ability, and finally to the mature cells of blood (Fig. 5.1).²⁶ In a recent modification in this scheme in mice, hematopoiesis matures through a common granulocyte-monocyte-lymphoid oligopotent progenitor that has not been identified in higher species.¹⁸ The strict division of common granulocyte-monocyte-erythroid-megakaryocytic progenitors and common natural killer (NK)/B and T cell progenitors occurs for human hematopoiesis, and likely applies to hematopoiesis in domestic species.

Lineage commitment typically follows expression of lineage-restricted transcription factors (Table 5.1). Induction of these transcription factors occurs through a combination of specific cytokine receptor and ligand interactions, less specific signal transduction pathways,

and an overlay of highly specific, permissive microenvironmental influences from stromal cells, endothelial cells, adipocytes, osteoblasts, ECM proteins, adherent cytokines, and trabecular bone.

Because of the short life-span of differentiated hematopoietic cells, mature blood cell production is an ongoing process; estimates suggest production of 1.5×10^6 cells/second in humans. It is unlikely that HSCs supply a continuous flux of cells from left to right as shown in Figure 5.1 because HSCs and multipotent cells are non-dividing or very slowly dividing during normal hematopoiesis. Newer evidence suggests that information determined from experiments in lethally-irradiated mice may not necessarily recapitulate normal or physiologically accelerated hematopoiesis and that there may be other homeostatic mechanisms that control hematopoiesis.²⁶

A somewhat confusing nomenclature has evolved around early committed hematopoietic progenitors based on *in vivo* experiments in mice. Splenic colonies

TABLE 5.1 Hematopoietic Transcription Factors^a**Early hematopoietic development**

SCL (TAL 1) (stem cell leukemia, T-cell acute lymphocytic leukemia-1)
 GATA-2
 LMO2 (Rbtn-2) (Lim finger protein)
 AML-1 (acute myeloid leukemia 1 protein)
 Tel
 Notch 1

Erythropoiesis

HIF-1 (Hypoxia-inducible factor-1)
 GATA-1
 EKLF-1 (erythroid kruppel-like factor 1)
 p45/NF-E2 (nuclear factor erythroid-2)
 STAT5a, STAT5b
 CBP/p300 (CREB binding protein/p300)
 SP1

Erythropoietic regulators

FOG-1 (friend of GATA-1)
 TRAP220 (thyroid hormone receptor-associated protein 220)
 BRG1 (Brahma-related gene 1)
 CBP/p300 (CREB binding protein/p300)

Myelopoiesis

Notch
 GATA-1
 C/EBP α (CCAAT/enhancer binding protein α)
 STAT5a, STAT5b

Granulocytopoiesis

RAR (Retinoic acid receptor)
 C/EBP α and C/EBP ϵ
 CBF (core binding factor)
 c-Myb
 STAT3
 NF κ B (nuclear factor of kappa B) – late precursor
 AML1 – precursor

Monocytopoiesis

EGR-1, EGR-2 (early growth response genes 1 and 2)
 Vitamin D receptor
 c-Fos, c-Fos
 PU.1
 RAR (retinoic acid receptor)
 C/EBP β and C/EBP ϵ
 MafB/c
 PU.1

Osteoclast differentiation from monocyte precursor

NF κ B

Megakaryocytopoiesis

Notch
 GATA-1
 PU.1
 Fli-1/NF-E2 (platelet production)
 FOG
 SCF (stem cell factor)
 STAT5a, STAT5b

Lymphoid

PU.1
 Ikaros
 STAT3, STAT5a, STAT5b, STAT4, STAT6

B-cell

E2A
 EBF (early B-cell factor)
 Pax 5
 NF κ B (progenitors and late maturation)

T-cell

HEB (TCF12 – T-cell factor 12)
 Notch/HES-1 (Hairy Enhancer of Split-1)
 NFAT (Nuclear Factor of Activated T cells) late maturation
 GATA-3
 TCF-1 (T cell factor 1)
 NF κ B (progenitors and late maturation)

Mast cell

GATA-2⁺
 Elf-1
 MITF (microphthalmia-associated transcription factor)

Eosinophiloipoiesis

STAT1, STAT3, STAT5a, STAT5b
 GATA-1

^aAll transcription factors are involved in early lineage commitment and exert their effects on committed progenitors unless otherwise stated.

that appear in lethally irradiated mice reconstituted with HSCs are called splenic colony forming cells (CFC-Ss), which have limited capacity for self-renewal. Whether similar cells occur in other species is unknown, because CFC-Ss are defined by specific experimental conditions in mice.

HEMATOPOIETIC PROGENITORS AND PRECURSORS

The term progenitors typically refers to cells whose presence is inferred from cytokine-driven differentia-

tion of colonies in culture, such as granulocyte-macrophage colony forming cells (CFU-GMs) and erythroid colony-forming units (CFU-Es), whereas the term precursors refers to stages of hematopoietic differentiation recognized by cytologic evaluation. HSCs, oligopotent stem cells, and committed progenitors appear similar to small lymphocytes, although they can be separated by flow cytometry based on expression of surface proteins.

Towards the right of Figure 5.1, clonogenic cell types exist putatively without any capacity for self-generation. However, mature cells types such as macrophages and mast cells may have substantial

self-generative properties. The initial model of hematopoiesis did not include the concept of plasticity.²⁶ In the current model, the concept of plasticity suggests that lineage fidelity and progressive restriction of proliferative capacity in the traditional model are not absolute. Plasticity is recognized as a property of both proliferation and lineage commitment. How plasticity is regulated under physiologic and pathologic conditions is not well understood. It is clear though that neither HSC nor any of the oligopotent progenitors have the unlimited capacity for self-generation possessed by embryonic stem cells.

SPECIES SPECIFICITY OF HEMATOPOIESIS

In contrast to detailed information about murine hematopoiesis,⁴⁵ there are few detailed descriptions about the structure and function of the bone marrow in domestic species. Stem cell function, the biochemical nature of the bone marrow microenvironment, hematopoietic cytokines and their receptors, and lineage specific and non-specific transcription factors are assumed to apply to species other than mice or humans.^{5,21} Studies have confirmed some common cytokines between domestic species and humans.^{14,24,25,27,28,30,31,38,39} Research involving animal models of human diseases, toxicology, or other unique diseases such as cyclic hematopoiesis in dogs has provided additional information about hematopoiesis in domestic animals.^{4,11,20,26} The gene sequences for many hematopoietic cytokines, receptors, and transcription factors for multiple species now are available in publically accessible databases so they can be assessed specifically by high density microarray or more broadly by quantitative reverse transcription polymerase chain reaction (qPCR).^{10,12,33}

Certain critical differences exist between human and murine hematopoiesis. Human HSCs express Flt3, the tyrosine kinase receptor for FLT3 ligand, whereas murine HSCs do not.¹⁸ However, both murine and human common lymphocyte progenitors (CLPs) express Flt3. Such differences are important because certain human acute myelogenous leukemias are associated with constitutive activation of Flt3.⁸ Whether other species express similar mutations governs the potential utility of therapy with Flt3-kinase inhibiting drugs. Although the hierarchical relationships observed in murine hematopoiesis are generally preserved in other species (Fig. 5.1), expression of a number of surface antigens at each developmental stage is different between mice and humans, and these and other differences may occur in other species. However, important similarities also have been recognized. Based on the recent molecular and genetic understanding of cyclic neutropenia in gray collie dogs and cyclic hematopoiesis in humans, some features of hematopoiesis appear similar. For example, allometric scaling correctly predicts granulopoietic cyclicity in mice (3 days), dogs (14 days), humans (19–21 days), and elephants (60 days).^{4,26}

THE BONE MARROW MICROENVIRONMENT

The heterogeneous cellular elements in Figure 5.1 are intimately associated with adipocytes, macrophages, endothelial cells, nerves, osteoclasts, osteoblasts, ECM, sinusoids, and cytokines, collectively termed the bone marrow microenvironment. Cell-cell and cell-ECM interactions are important in the regulation of cell proliferation and differentiation of hematopoietic cells.⁹ These interactions involve receptors and integrins, which are regulated by growth factors, cytokines, and transcription factors. The ultrastructure of the interactions between hematopoietic cells, stromal cells and non-cellular components of the bone marrow microenvironment were described in detail in 1978,²⁹ but the molecular mechanisms have more recently been characterized.

The best characterized integrin on HSC is very late antigen 4 (VLA-4 or $\alpha 4 \beta 1$) which binds to fibronectin in the ECM as well as to vascular cellular adhesion molecule 1 (VCAM-1) on adjacent stromal cells. The binding of VLA-4 to fibronectin mediates adhesion of HSC and progenitors to the microenvironment, homing of circulating HSC and progenitors to specific areas within the bone marrow, and signal transduction. The Rho guanosine triphosphatases (GTPases), Ras-related C3 botulinum toxin substrate 1 (Rac1), and Rac2 also are key regulators of adhesion and migration of cells in the hematopoietic microenvironment.

The bone marrow microenvironment has highly specialized and integrated microanatomic functional units called niches. The two most clearly elucidated hematopoietic niches are the HSC niche (see Chapter 3) and the erythroblastic islands (Figs. 5.2 and 5.3; see Chapter 6).¹

The Hematopoietic Stem Cell Niche

Survival, proliferation, and differentiation of HSCs depend on their spatial and functional relationships with the cells and ECM of the microenvironment.²⁹ HSCs are enriched at sites adjacent to the endosteal surface of bone. A specialized subset of activated osteoblasts display ligands and receptors that facilitate homing and transient docking of HSC, and regulate slow cycling or rapid mobilization of HSCs as needed.¹ The interaction between stromal-derived factor 1 (SDF-1 or CXCL12) elaborated by osteoblasts and CXC chemokine receptor 4 (CXCR4), its cognate chemokine receptor on HSC, is central to this HSC niche. SDF-1 recruits quiescent progenitors, participates in their cycling and survival, and sensitizes them to further synergistic action of cytokines, thus contributing to hematopoietic homeostasis under both physiologic and stress conditions. HSC function also is regulated by osteoblasts through parathyroid hormone (PTH), the Notch signaling pathway, and interactions between angiopoietin 1 (Ang1) and its tyrosine kinase with immunoglobulin-like and EGF-like domains 2 (Tie2) receptor.⁶ Disruption of this HSC niche may result in abnormal cell mobilization and contribute to extramedullary infiltration in leukemia.

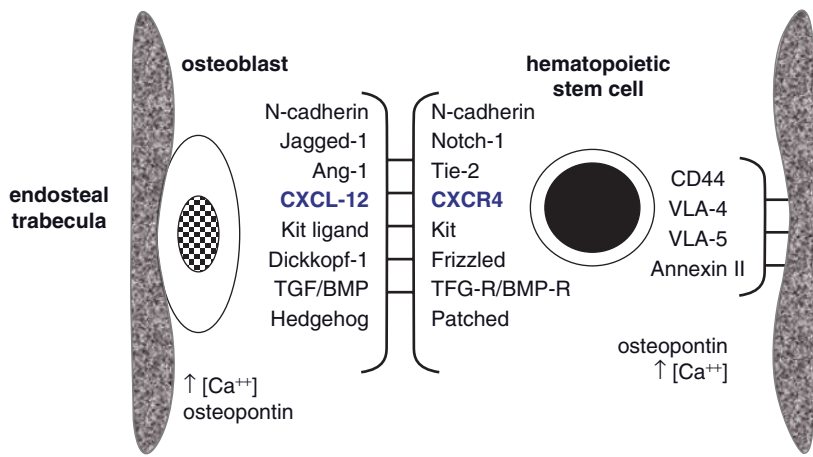


FIGURE 5.2 Hematopoietic stem cell niche (reciprocal molecular interactions). The relationship between a specialized subset of osteoblasts and the hematopoietic stem cell is shown, with reciprocal molecular interactions depicted between the two cell types, and between these cell types and endosteal bone.

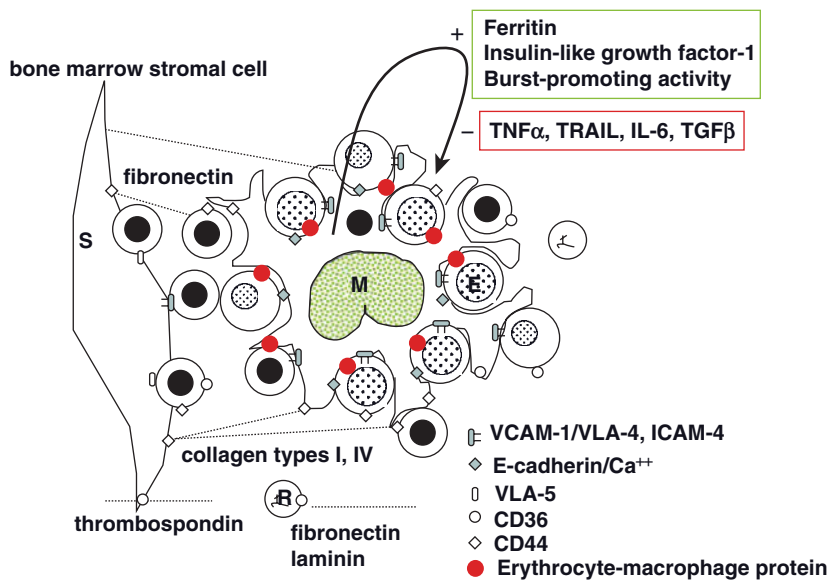


FIGURE 5.3 The erythropoietic niche. The erythroblastic island is illustrated with rubriblasts of graded levels of maturity surrounding macrophage (M) cytoplasm. Specific molecular interactions between macrophages and rubriblasts, between the erythroblastic island and marrow stromal cell(s), and of all these cellular elements with marrow stroma are shown. Autocrine cytokine loops involved in stimulation of erythropoiesis (green bordered box) and in the negative regulation of erythropoiesis (red bordered box) are shown. Dotted lines indicate extracellular matrix components. Arrow indicates factors elaborated by macrophages that influence growth and maturation of rubricytes.

The Erythropoietic Niche

Erythropoiesis occurs in distinct niches called erythroblastic islands that consist of a central macrophage surrounded by a ring of developing erythroblasts in bone marrow, fetal liver, and spleen, and even in long term marrow cultures.¹⁷ The macrophage contributes important signals to developing rubriblasts, phagocytoses expelled metarubricyte nuclei, and transfers iron to developing rubriblasts. Unlike megakaryocytes that localize exclusively to marrow sinusoids, erythroblastic islands are located throughout the marrow.⁹

Adhesion molecules mediating important structural and functional interactions between developing erythroid cells and central macrophages include VCAM-1/VLA-4, $\alpha 4\beta 1$ /VLA-4, intercellular adhesion molecule-4 (ICAM-4)/ αv , and E-cadherin. For example, ICAM-4 is postulated to enable reticulocytes to detach from central macrophages, allowing them to enter the circulation.⁹ Laminin and fibronectin and their receptors expressed on late-stage rubriblasts also are key components in differentiation of reticulocytes. VLA-4 and VLA-5 are

involved in binding of erythroid burst forming units (BFU-Es) to hematopoietic stromal cells. Expression of these adhesion molecules is highest on BFU-Es and erythroid colony forming units (CFU-Es) and is progressively lost during erythroid maturation. Hemonectin and collagen type I support binding of BFU-Es in vitro, and a role for tenascin-C has been inferred from knock-out mice. Hemonectin is absent from anemic mice with Kit ligand or *c-kit* deficiency.

The surface antigen cluster differentiation 44 (CD44) is highly expressed on almost all hematopoietic cells in bone marrow and is responsible for interaction of these cells with collagen types I and IV, fibronectin, and hyaluronate.⁴⁴ Reticulocytes express only low levels of a few surface adhesion receptors, such as CD36 (thrombospondin receptor) and VLA-4. Thrombospondin serves as an adhesive ligand for committed progenitors including colony forming units-granulocyte erythroid monocyte megakaryocyte (CFU-GEMM) and BFU-E. Adhesion molecule interactions of reticulocytes with bone marrow stromal cells and ECM may facilitate their egress into bone marrow sinuses. Mature red blood

cells (RBCs) do not express adhesion molecules under normal conditions.

CYTOKINES AND CYTOKINE SIGNALING IN HEMATOPOIESIS

Hematopoiesis is the cumulative result of intricately regulated signaling pathways mediated by soluble cytokines and their receptors (Table 5.2).² Evaluation of cytokine-receptor interactions in hematopoiesis has largely been achieved through creation of gene-deleted mice and conditional knock-out mice, the phenotypes of which range from severe lethal embryonic, fetal, and neonatal defects to redundant null phenotypes.⁷ These mice have provided much of our current insight into the regulation of physiologic and pathologic alterations in hematopoiesis.

Most hematopoietic cytokine receptors are multiple subunit complexes, with the exception of those that signal through a single chain, such as erythropoietin (EPO), granulocyte-macrophage colony-stimulating factor (GM-CSF), and thrombopoietin (TPO). Hematopoietic cytokines are approximately 200 amino acids in length and carry a conserved sequence of tryptophan-serine-X-tryptophan-serine (W-S-X-W-S) in their extracellular domain, which functions as part of the ligand binding domain.

Cytokine receptors contain docking regions for Janus tyrosine kinases (JAK1, JAK2, JAK3, TYK2) in their cytoplasmic termini, that when attached to the ligand-

bound form of the cytokine receptor, recruit JAKs which then autophosphorylate. The JAK kinase then phosphorylates tyrosine residues on a specific signal transducer and activator of transcription (STAT) protein, which is STAT1, 3, or 5 for most hematopoietic cytokine receptors. JAKs contain a catalytic Janus homology (JH) 1 domain and a JH2 catalytic-like but inactive domain critical to the ability of JAKs to regulate themselves and to mediate cytokine-induced responses. Src kinase activation of STATs also is important for myeloid cell proliferation.

In unstimulated cells, STATs are present as cytoplasmic monomers in the unphosphorylated state. Phosphorylation by JAK kinases leads to dimerization through reciprocal interactions of SH2 domains with phosphotyrosine residues, and thereby activation of STAT, which then translocates to the nucleus.¹⁵ STATs are transcription factors that prevent apoptosis or positively regulate prosurvival genes of late progenitor and early precursor cells.¹⁵ Although there are numerous STAT responsive genes in many different cell types, STAT regulation of hematopoietic precursors occurs in a cell-type restricted manner.

Lineage-committed colony-forming cells respond to cytokines in an absolute lineage-restricted fashion. The specificity of hematopoietic cytokines is determined by progenitor and precursor cell expression of their cognate receptors. For example, rubriblasts express EPO receptors but myeloblasts do not, which is different from the relatively promiscuous expression of JAK/STAT pathways. Given the importance of JAK-phosphorylation events in driving proliferation of hematopoietic precursors, it is not surprising that mutations leading to constitutively active JAK2 result in myeloproliferative disorders and leukemia. In addition to the prominent role of JAK-STAT interactions in hematopoiesis, the functional involvement of Ras and phosphoinositol 3 kinase (PI3K) pathways also has been shown following interleukin (IL)-3/IL-5 and GM-CSF stimulation of bone marrow cultures *in vitro*.²

Negative Regulation of JAK-STAT Signal Transduction

Suppressors of cytokine signaling (SOCSs) are a family of proteins that regulate the strength and duration of the hematopoietic cytokine-driven signaling cascade.⁴² They are transcriptionally induced by JAK-STAT signaling. SOCS proteins contain src homology (SH) 2 domains and a SOCS-box which mediate binding to cytokine receptors and associated JAKs, and attenuate signal transduction directly. In addition to their transcriptional induction by hematopoietic cytokines and subsequent self-limiting stimulation of hematopoiesis, other cytokines including tumor necrosis factor- α (TNF- α), IL-1, and Toll-like receptor ligands (e.g. lipopolysaccharide) also induce SOCS expression, providing negative regulation for granulocyte colony stimulating factor (G-CSF) signaling.

Another level of regulation is provided by phosphatases. The signaling and subsequent hematopoiesis

TABLE 5.2 Hematopoietic Cytokines^a

Lineage/Function	Key Hematopoietic Cytokine
Early hematopoiesis	Stem Cell Factor (SCF) Interleukin 3 (IL-3) Wnt ligands (Jagged) and receptors Kit ligand SDF-1/FGF-4
Common myeloid progenitor/myelopoiesis	Stem cell factor (SCF) Thrombopoietin (TPO)
Erythropoiesis	Erythropoietin (EPO)
Megakaryocytopoiesis	Thrombopoietin Interleukin-6 (IL-6)
Lymphocytopoiesis	IL-7
T cells	IL-7, IL-2
NK cells	IL-7, IL-15
B cells	IL-4
Regulatory	Transforming growth factor- β (TGF- β) Bone morphogenetic protein (BMP) Activin

^aNote that this table oversimplifies the actions of these cytokines. With further refined analyses of murine models it has become apparent that even lineage restricted cytokines such as EPO exert pleiotrophic effects within and external to the hematopoietic system.

induced by phosphorylated dimers of STAT proteins is terminated by removal of phosphates from STAT tyrosines by three specific protein tyrosine phosphatases.

Transforming growth factor- β (TGF- β) is perhaps the most potent endogenous negative regulator of hematopoiesis.¹⁹ TGF- β suppresses expression of the SCF receptor, the response of progenitors to SCF, and cell cycle progression of progenitors. The expression of receptors for TGF- β on primitive hematopoietic progenitors and subsequent stages of maturation suggests a broad role for this cytokine. Negative regulation of erythropoiesis by TNF- α , TNF-related apoptosis inducing ligand (TRAIL), IL-6, and TGF- β occurs when chronic inflammatory disease increases systemic and local bone marrow concentrations of these cytokines (see Chapter 37).⁹

EVALUATION OF HEMATOPOIETIC FUNCTION

Hematopoiesis is studied to gain insight into mechanisms of cytopenias, leukemias, and other pathophysiologic responses. Evaluation of hematopoiesis begins with careful examination of peripheral blood and is complemented by cytologic or histologic assessment of bone marrow. Short and long term *in vitro* culture of bone marrow-derived cells, genetically modified mice, animal models of retarded and accelerated hematopoiesis, syngeneic and xenogeneic hematopoietic stem cell transplantation, retroviral-mediated gene transfer, and gene therapy also have been used to study hematopoiesis.⁴³

Gene-deleted and transgenic mice provide a special challenge to the hematologist because these mice frequently die during the embryonic, fetal, or early neonatal period. Peripheral blood examination may still provide valuable information. A 3 μ L volume of heart blood obtained with a fine gauge needle is sufficient to prepare a blood smear; as little as 20 μ L of heart blood may be diluted with 2 mg/mL ethylenediaminetetraacetic acid (EDTA) in saline and analyzed with an electronic counter. Potential artifacts of dilution of small quantities can be overcome by comparing results from treated or genetically altered mice with similarly diluted volumes from control or wild type mice.

Bone Marrow Evaluation in Mice

Direct examination of bone marrow complements information gained from assessment of blood. The technique of bone marrow collection and analysis in mice is described in Chapter 132. In fetal mice, which lack developed medullary hematopoiesis, cytologic examination of liver imprints for hematopoietic precursors is useful in assessing early hematopoietic function.

Evaluation of Hematopoiesis with Bone Marrow Culture

Hematopoietic interactions are best evaluated *in vitro* where culture systems permit evaluation of effects of individual cytokines, growth factors or their regulators,

and combinations of factors (see Chapter 133). Target gene expression may be transiently induced by gene transfection or inhibited by transfection with antisense RNA. The significance of *in vitro* studies must be confirmed *in vivo*. For example, deficiency of hematopoietic cytokines or growth factors which would predictably have severe phenotypes based on *in vitro* work, such as IL-2 and GM-CSF, have much milder hematologic phenotypes than expected (i.e. failure to develop lymphopenia and neutropenia), underscoring the redundancy and pleiotrophy which characterize many of these factors.

Bone marrow cells can be cultured from aspirates or core samples obtained from diseased or healthy animals. These cells are cultured in semisolid methylcellulose-based media with cocktails of cytokines, EPO, transferrin, bovine fetal serum, and albumin. Whereas EPO is active across species and has been cloned from a variety of species, other cytokines have more limited cross-species effects. When a species-specific cytokine is not available, conditioned spleen cell media obtained from phytohemagglutinin, endotoxin, or phorbol ester-stimulated cells may serve as a useful source of cytokines. Conditions for culture of bone marrow progenitors from dogs, cats, sheep, cattle, horses, chickens, and other species have been described.^{13,16,40,37} In general, methodologies applicable to murine and human culture systems apply to those of other species, when care is taken to ensure the quality of collected bone marrow specimens and species-specific reagents or appropriate substitutes are used. Short-term bone marrow cultures from mice, rats, and dogs are routinely performed to assess the potential toxicity of xenobiotics and define hematopoietic phenotypes of genetically altered animals.³⁷ These culture systems may be used to study hematopoiesis by the addition of individual cytokines, growth factors or their regulators, or combinations thereof. Alternatively, neutralizing antibodies to these components or small molecules (<1000 kDa) produced by medicinal chemistry can be used to dissect regulatory pathways. Similarly, hematopoietic progenitors from clinical cases can be studied *in vitro* by direct culture following aspiration biopsy. Plasma or plasma components from animals with bone marrow disorders may be added to bone marrow cells of clinically normal animals to study the nature of potential humoral myelosuppressive activities.

Long term cultures of hematopoietic cells on established stromal cell layers (Dexter cultures) more closely recapitulate the hematopoietic microenvironment and allow long term survival of pluripotent HSC. These culture systems were used to identify the stromal cell defect (defective SCF production) in *Sl/Sl^d* mouse (Steel anemia) and the complementary defect in *W/W^v* (white spotted) mouse (SCF receptor alternatively known as *c kit*). Long term bone marrow cultures have been described for dogs^{36,37} and sheep.²²

Lethal Irradiation and Bone Marrow Transplantation

Lethally irradiated mice receiving bone marrow or hematopoietic cells with a capacity for partial or

complete renewal of hematopoiesis develop large splenic foci of extramedullary hematopoiesis thought to be initiated by a single pluripotent stem cell. These colonies are grossly visible after 8–14 days, creating hemispherical distortions of the splenic contour which may be visually enumerated.³⁴ These CFU-S have since been found to represent a multipotent committed progenitor cell rather than the pluripotent stem cell as originally hypothesized. Irradiation and stem cell transplantation methodologies are described in detail for dogs.³⁷

Genetically Altered Mice

Through technology that permits homologous recombination of partially homologous, generally noncoding, genetic sequences in cultured murine stem cells, mice deficient in the protein product of specific target genes can be created. This technology has been extensively exploited to study the function of individual hematopoietic cytokines, growth factors, their cognate receptors, and hematopoietic transcription factors.³³ Without this technology, the study of transcription factors associated with primitive oligopotent progenitors (Tables 5.1 and Table 5.2) would not have been possible. The hematologic evaluation of mouse embryos or fetuses is a critical part of the phenotypic assessment of knockout mice. By rendering gene-deleted mice (–/–) transgenic (+/+) for a deficient gene under the control of an inducible or repressible tissue-specific promoter, the function(s) of a gene in adults, for which deletion otherwise results in nonviable fetuses, can be examined. Using the Cre-loxP system, genes may be removed or reactivated in a tissue specific manner in the adult mouse.²³

The development of methods to transplant human hematopoietic cells into severe combined immunodeficient (SCID) mice (scid-hu) has provided an experimental tool with potential use in modeling leukemias, infectious diseases, autoimmune diseases, and a variety of primary nonneoplastic bone marrow disorders. Potential deficiencies of this model include incomplete cross-species functionality between murine cytokines and cytokine receptors and human receptors and ligands, since the absence of human bone marrow stroma in mice transplanted with human hematopoietic cells creates a chimeric hematopoietic microenvironment. Impaired passage of mature cells into and out of the bone marrow or circulation may occur when adhesion molecules and their receptors on leukocytes, platelets, and endothelial cells fail to recognize their respective murine or human counterparts.

Models of Accelerated Hematopoiesis

Several methods have been used to accelerate hematopoiesis. The intraperitoneal administration of a single 150 mg/kg dose of 5-fluorouracil or appropriate dosing regimen of cyclophosphamide completely depopulates murine bone marrow. Treatment with 5-fluorouracil spares the slowly cycling or noncycling stem cells.⁴⁶ Recovery of hematologic parameters is first detectable 5 days post-treatment, with complete recovery occur-

ring after 15–20 days. This model has been used extensively to study accelerated hematopoiesis in gene-deleted mice with apparently normal basal hematopoiesis or in mice treated with agents that do not alter basal hematopoietic function. In this manner, the immunosuppressant rapamycin was shown, by virtue of its ability to inhibit signal transduction of a variety of proliferation-inducing cytokines, to have potential liability when administered in concert with cytotoxic agents.³⁵ Accelerated erythropoiesis is readily induced in rodents by a single 60 mg/kg intraperitoneal injection of phenylhydrazine. This regimen produces Heinz body hemolytic anemia with approximately 30% decrease in hematocrit after 2 days and a robust reticulocytosis by 5 days post-treatment.⁴¹ Alternatively, a fixed volume of blood (up to 3% of body weight) can be removed from a rodent and replaced with intraperitoneal saline, stimulating erythropoiesis. To mobilize granulocytes and accelerate granulopoiesis, Bacto-tryptone (a potent chemotactic casein digest, 1 mL of a 10% solution) is administered intraperitoneally. A similar effect is achieved with intravenous G-CSF, which generally is species cross-reactive, at a dose of 5 µg/kg.³

Lessons learned from evaluation of hematopoiesis in genetically-altered rodents may be extended to the study of hematopoiesis in domestic species and provide a better understanding of dysregulation in primary diseases of bone marrow. The availability of specific reagents for domestic species and accessibility to bone marrow culture, flow cytometry, and polymerase chain reaction (PCR) technologies permit increasingly sophisticated examination of bone marrow function, providing greater insight into abnormalities recognized by traditional hematologic methods.

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Erythropoiesis

CHRISTINE S. OLVER

Definitions

Stem Cell to Erythrocyte

General

Development and Maturation of Reticulocytes

Cell Surface Molecules on Developing Erythroid Cells

Reticulocytes in Veterinary Species

Molecular Control of Erythropoiesis

Cytokines and Growth Factors Regulating Erythropoiesis

Transcription Factors

Pleiotropic transcription factors

Erythroid-specific transcription factors

Other Molecules

Negative Regulators of Erythropoiesis

Microenvironment

Molecules Involved in Adhesion

Erythrokinetics

Acronyms and Abbreviations

BFU-E, burst-forming unit erythroid; CD, cluster of differentiation; CFU-E, colony-forming unit erythroid; CMP, common myeloid progenitor; DNA, deoxyribonucleic acid; EKLF, erythroid Kruppel-like transcription factor; EMP, erythrocyte macrophage protein; EPO, erythropoietin; EPOR, erythropoietin receptor; FasL, Fas ligand; FOG-1, friend of GATA; GATA1 and GATA2, GATA binding protein 1 and 2; GM-CSF, granulocyte/macrophage colony stimulating factor; HIF-1, hypoxia inducing factor-1; HSC, hematopoietic stem cell; ICAM-4, intracellular adhesion molecule-4; IGF-I, insulin like growth factor I; IL, interleukin; IFN- γ , interferon gamma; JAK2, Janus kinase 2; kDa, kilodalton; mRNA, messenger ribonucleic acid; MEP, megakaryocyte/erythroid progenitor; RBC, red blood cell; RNA, ribonucleic acid; RT-PCR, reverse transcriptase polymerase chain reaction; TNF, tumor necrosis factor; SCF, stem cell factor; SCL, stem cell leukemia; SOCS, suppressor of cytokine signaling; STAT, signal transducers and activators of transcription; TNF, tumor necrosis factor; TPO, thrombopoietin; TRAIL, TNF-related apoptosis inducing ligand; VCAM, vascular cell adhesion molecule; VLA-4, very late antigen-4.

Erythropoiesis is the proliferation and progressive differentiation of hematopoietic stem cells (HSCs) (see Chapters 3 and 5) into hemoglobinized, red blood cells (RBCs). In normal animals, erythropoiesis and RBC mass are regulated by cellular oxygen levels. RBCs originate from HSC in a stepwise manner, wherein each step includes cell division and differentiation, and is initiated and regulated by specific humoral, microenvironmental, cell surface, and transcription factors. First, HSCs commit to erythroid lineage to form erythroid progenitor cells, which resemble lymphocytes. Second, erythroid progenitors differentiate into erythroid precursors that can be identified morphologically. Precursor cells undergo a series of division and differentiation steps until the nucleus is extruded (in mammals) and reticulocytes are formed. This chapter describes erythropoiesis in adult animals, and is a synthesis of knowledge gained primarily from studies in

mice, rats, and humans. Species-specific information is provided when available.

DEFINITIONS

Basal erythropoiesis is erythropoiesis that occurs to replace naturally senescent RBCs and therefore maintain normal RBC mass. Stress erythropoiesis refers to erythropoiesis that occurs in response to an anemic stimulus such as blood loss, hemolysis, or pregnancy.

STEM CELL TO ERYTHROCYTE

General

HSCs are multipotent and can divide into copies of themselves or into cells committed to specific blood cell

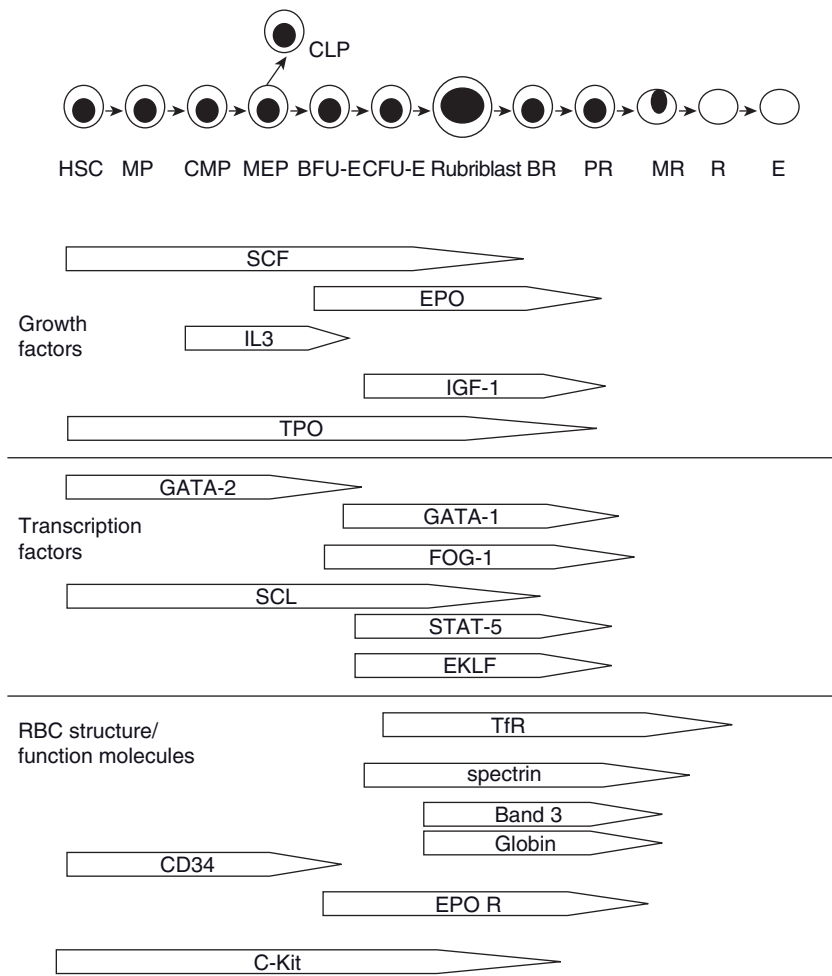


FIGURE 6.1 Approximate timing of the response to certain growth factors, activation of transcription factors, and expression of erythroid specific molecules in erythropoiesis.

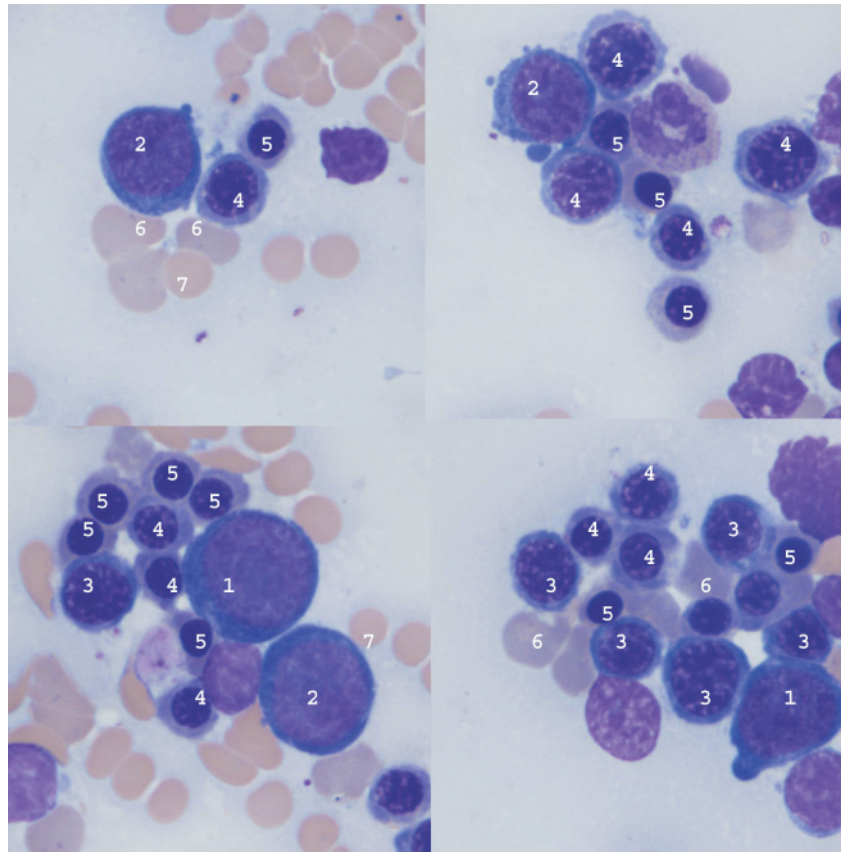
Abbreviations: BFU-E, burst forming unit erythroid; BR, prorubricyte/basophilic rubricyte; CFU-E, colony forming unit erythroid; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; E, RBC; EKLF, erythroid Kruppel-like factor; EPO, erythropoietin; EPOR, erythropoietin receptor; FOG-1, friend of GATA-1; HSC, hematopoietic stem cell; IGF-1, insulin-like growth factor-1; IL3, interleukin 3; MEP, megakaryocytic/erythroid progenitor; MP, multipotential progenitor; MR, metarubricyte; PR, polychromatophilic rubricyte; R, reticulocyte; Rub, rubriblast; SCF, stem cell factor; SCL, stem cell leukemia gene; STAT, signal transducer and activator of transcription; TfR, transferrin receptor; TPO, thrombopoietin.

lineages. A multipotent HSC (Fig. 6.1) first differentiates into a common myeloid progenitor (CMP), which is then capable of differentiating into any blood cell except lymphocytes.² The CMP has limited self-renewing capacity, and, depending on environmental signals, is capable of differentiating into a granulocyte/macrophage progenitor or a megakaryocyte/erythroid progenitor (MEP). CMPs and MEPs in humans and mice can be identified by their specific cell surface phenotype.² Differentiation from CMP to MEP is associated with expression of various gene-specific transcription factors, growth factor receptors, and functional proteins, and is influenced by a variety of growth factors. Gene-specific transcription factors are predominantly activators of transcription and usually function as a complex of proteins that bind to specific deoxyribonucleic acid (DNA) sequences.

MEPs differentiate into burst-forming units-erythroid (BFU-Es). Although BFU-Es are programmed to differentiate only into erythroid cells, they resemble lymphocytes microscopically. These cells are identified by their growth from a single cell to several thousand cells in culture (see Chapter 133). The colony-forming unit-erythroid (CFU-E) differentiates from BFU-E. In culture, each CFU-E divides into 8–32 cells. Thus, BFU-Es have a higher proliferative potential than the more differenti-

ated CFU-E. These progenitor cells differentiate into precursor cells that can be recognized as erythroid cells based on morphologic characteristics. The earliest precursor is a rubriblast (Fig. 6.2). This is a large cell with deeply basophilic cytoplasm; a round, centrally located nucleus; finely granular but deeply staining nuclear chromatin; and one or more prominent nucleoli (see Chapter 132). The deeply basophilic cytoplasm helps distinguish early erythroid from early myeloid precursors. Rubriblasts undergo a series of differentiation steps that result in a progressive decrease in cell size, a gradual increase in cytoplasmic hemoglobin concentration, and gradual condensation of nuclear chromatin. The prorubricyte is similar to the rubriblast except that it does not contain a nucleolus. Subsequent stages are called basophilic rubricytes, polychromatophilic rubricytes, and metarubricytes. These cells have increasing amounts of hemoglobin, which affects the staining quality of the cytoplasm. Basophilic rubricytes have the lowest hemoglobin concentration, and therefore still have deeply basophilic cytoplasm. Polychromatophilic rubricytes have cytoplasm that varies from blue-gray to gray, depending on hemoglobin concentration. Metarubricytes have the most hemoglobin, and therefore have gray to reddish cytoplasm. Metarubricytes also have dense, homogeneous nuclear chromatin. At

FIGURE 6.2 The various stages of erythroid precursors are shown. The progression or maturation is described in the text. (1) Rubriblast; (2) prorubricyte; (3) basophilic rubricyte; (4) polychromatophilic rubricyte; (5) metarubricyte; (6) reticulocyte; (7) RBC.



this point the nucleus is extruded to produce polychromatophilic RBCs, which stain as reticulocytes with vital stains such as new methylene blue. All of the precursors except metarubricytes are capable of division.

The nomenclature for erythroid precursors in humans and mice differs from that used in veterinary medicine. The earliest erythroid precursor is called a proerythroblast and subsequent cells are called basophilic erythroblasts, polychromatophilic erythroblasts, and orthochromatic erythroblasts (Table 6.1).

Development and Maturation of Reticulocytes

Nuclear expulsion from metarubricytes is associated with changes in intermediate filaments and microtubules that result in rearrangement of the membrane cytoskeleton.²⁴ Extruded nuclei are rapidly ingested by bone marrow macrophages. Reticulocytes then exit the bone marrow, although the exact mechanism is unknown. Reticulocytes contain residual mitochondria, Golgi membranes, ribosomes, and microtubular components, and can have approximately 35% more volume than mature RBCs.⁵⁰ During maturation in the circulation, reticulocytes gain hemoglobin and lose organelles, membrane surface area, volume, and numerous cell surface proteins. Cell surface proteins are lost in the form of exosomes, or membrane blebs, that contain proteins specifically selected for removal (see Chapter 22).²⁰

Stress reticulocytes are released from the bone marrow during times of erythropoietic stress.³³ Stress

TABLE 6.1 Nomenclature of Erythroid Precursors

Veterinary Terminology	Human/Mouse/Research Terminology
Rubriblast	Proerythroblast
Prorubricyte	Basophilic erythroblast
Basophilic rubricyte	Basophilic erythroblast
Polychromatophilic rubricyte	Polychromatophilic erythroblast
Metarubricyte	Orthochromatic erythroblast
Reticulocyte	Reticulocyte
Erythrocyte	Erythrocyte

reticulocytes are larger and less mature than normal reticulocytes, and contain more mitochondria, ribosomes, and other organelles. Their membranes are more mechanically rigid and unstable, and they possess adhesive proteins that are normally lost before release.

Cell Surface Molecules on Developing Erythroid Cells

Erythroid cells express a variety of cell surface molecules, some of which are developmentally regulated.^{29,32,41,51} A summary of important erythroid cell surface molecules is presented in Table 6.2.

Reticulocytes in Veterinary Species

The number of reticulocytes circulating during health varies among species (see Chapter 136).¹⁸ Some species

TABLE 6.2 Erythroid Cell Surface Molecules

Molecule	Function	Expression
CD34	Possibly signal transduction	BFU-E
CD36 (platelet glycoprotein IV)	Binds to thrombospondin	CFU-E to mature erythrocyte (decreasing)
CD71	Transferrin receptor (iron intake)	CFU-E to reticulocyte
CD49d/CD29 (VLA-4)	Binds to thrombospondin, VCAM-1, fibronectin	CFU-E to reticulocyte
CD105 (endoglin)	TGF- β receptor	BFU-E to rubriblasts
CD117 (c-kit)	Signal transduction	BFU-E to basophilic rubricyte
CD232 (ICAM-4)	Binds to integrins	CFU-E to reticulocyte

have circulating reticulocytes in health (dogs, cats, rodents, rabbits, pigs, guinea pigs), whereas others (cows, goats, sheep) have circulating reticulocytes only during a regenerative response. Horses, with rare exceptions,⁵⁵ do not have circulating reticulocytes even during a regenerative response.⁹

MOLECULAR CONTROL OF ERYTHROPOIESIS

Proliferation and differentiation of erythroid cells include cell divisions accompanied by a serial reduction of proliferative ability and increased erythroid-specific gene expression. The general sequence of molecular events for erythroid proliferation and differentiation is: (1) growth factor binding to its receptor; (2) activation of membrane-associated kinases; (3) phosphorylation of the cytoplasmic portion of the receptor and of other intermediate molecules; (4) activation of transcription factors; and (5) transcription factor-dependent synthesis of either additional transcription factors or erythroid-specific functional proteins, depending upon the stage of erythropoiesis. Examples of erythroid specific functional proteins include RBC specific spectrins, globins, ankyrin, band 4.1, and glycophorins (cell surface molecules).²⁴ Details of the molecular control of self-renewal, proliferation, and differentiation are complex and not yet entirely understood, although Zhu and Emerson provide an excellent review.

Cytokines and Growth Factors Regulating Erythropoiesis

Erythropoietin (EPO) is the principal hormonal regulator of erythropoiesis. EPO is a 34.4 kilodalton (kDa) glycoprotein (165 amino acids) that is produced primarily by peritubular interstitial fibroblasts of the kidney in adult animals. A small amount of EPO is produced

in the liver during anemia.²³ Low tissue oxygen tension induces *EPO* gene expression through transcriptional activation and also induces *EPO* messenger ribonucleic acid (mRNA) stabilization.^{8,10,57} Hypoxia up-regulates *EPO* by allowing accumulation of hypoxia inducible factor-1 (HIF-1). This transcription factor, along with cofactors, binds a responsive element in the 3' flanking region of the *EPO* gene. In severe hypoxia, production of *EPO* is increased up to 1,000-fold.¹⁰ *EPO* production by erythroid progenitors themselves also has been shown using reverse transcriptase polymerase chain reaction (RT-PCR), suggesting autocrine stimulation.⁴⁴ *EPO* is highly conserved among species, and recombinant human *EPO* can support proliferation and differentiation of erythroid progenitors from a variety of species including horses and cats (see Chapter 133).

The *EPO* receptor (EPOR) is an integral membrane protein with seven tyrosine phosphorylation sites in its cytoplasmic domain. Each of these domains appears to regulate different aspects of erythropoiesis. Some phosphorylation sites may be dispensable for steady-state erythropoiesis, but required for stress erythropoiesis.^{26,56} EPOR-deficient mice die in utero with anemia and deficient hepatic erythropoiesis. Signaling through the EPOR is mediated by pre-bound Janus kinase 2 (JAK2), a phosphokinase. JAK2 phosphorylates several tyrosine residues in the cytoplasmic domain of the EPOR, which results in recruitment of a number of cytoplasmic signaling proteins. Several pathways critical to survival and differentiation of erythroid progenitors and precursors are then activated. The signal transducers and activators of transcription (STAT) 5 pathway, which results in up-regulation of the Bcl-x_L anti-apoptotic protein, is an important example of one of these pathways. The EPOR is expressed on early cluster of differentiation 34-positive (CD34+) hematopoietic progenitors and decreases gradually as erythroid differentiation progresses (Fig. 6.1). Neither *EPO* nor EPOR is required for generation of BFU-E or CFU-E, but both are required for development of later stages of erythroid precursors and for production of mature RBCs.⁵⁷

Interleukin-3 (IL-3) is a multilineage cytokine produced by macrophages, activated T lymphocytes, mast cells, eosinophils, and bone marrow stromal cells. IL-3 acts on HSC and progenitor cells to induce proliferation and self renewal. It also primes progenitor cells to respond more vigorously to *EPO*.¹

Stem cell factor (SCF), also known as Steel factor, mast cell growth factor, and c-kit ligand, is expressed by bone marrow stromal cells and acts through its receptor, c-kit (CD117).⁶ Its mRNA is also expressed in BFU-E, suggesting a potential autocrine or paracrine function.²⁷ SCF is important for stem cell maintenance,³⁰ and is synergistic with other cytokines to produce differentiated cells. SCF exists as a soluble form and a membrane associated form.⁴⁶ SCF markedly enhances the activity of *EPO*, allowing for proliferation and differentiation of progenitors and precursors. Mice with deficiencies in the membrane-associated form have macrocytic anemia, in spite of having the secreted form of SCF.

Granulocyte-macrophage colony stimulating factor (GM-CSF), produced by T cells, stimulates production of primarily neutrophils, monocytes, and eosinophils. GM-CSF may enhance erythroid progenitor development, although GM-CSF knockout mice are hematologically normal.

Thrombopoietin (TPO) is a 45–70 kDa (depending on glycosylation) glycoprotein produced in the liver, kidney, skeletal muscle, and bone marrow stroma.⁶ It appears to be required for maintenance of HSCs in G_0 and therefore their self-renewal capacity.³⁸ TPO also acts as a co-stimulatory agent with EPO to promote BFU-E growth in vitro and to support proliferation and maintenance of erythroid progenitor cells in vivo.^{3,21,49,58,59}

Insulin-like growth factor-1 (IGF-1) and insulin both enhance the growth and differentiation of erythroid cells. IGF-1 decreases apoptosis of progenitors and enhances heme synthesis and nuclear condensation and enucleation.³¹

Glucocorticoids enhance erythroid colony formation in vitro⁷ and in vivo⁴ by stimulating erythroid progenitor proliferation and inhibiting differentiation.⁵⁴ Glucocorticoids act through a nuclear glucocorticoid receptor that subsequently directly affects transcription by binding to glucocorticoid response elements in DNA.⁵⁵

Humoral inhibitors of erythropoiesis include tumor necrosis factor (TNF), TNF-related apoptosis inducing ligand (TRAIL), IL-1, and interferon gamma (IFN- γ).⁴² TNF and IL-1 not only inhibit erythroid progenitors, but also suppress EPO secretion. Immature erythroid cells are susceptible to cell death due to TNF and IFN- γ up-regulation of “death” receptors.¹²

Transcription Factors

Several transcription factors are activated during RBC development as a result of binding of hematopoietic cytokines to cell surface receptors. Activated transcription factor then translocates to the nucleus to enhance transcription of specific genes. The DNA that is transcribed is either an erythroid-specific structural or functional gene (e.g. spectrin and globin) or a gene encoding a protein that is involved in initiation of the next step in differentiation (e.g. another transcription factor).

Pleiotropic Transcription Factors

The stem cell leukemia (*SCL*) gene is a pleiotropic (not erythroid-specific) transcription factor that is crucial for hematopoiesis; *SCL* null mice die in utero at day 8 without any blood cell formation.^{37,45} *SCL* also enhances erythroid proliferation and differentiation² while repressing myeloid differentiation.

GATA binding protein 2 (GATA2) is expressed in early HSCs. It appears to be necessary for proliferation and survival of stem cells but dispensable for terminal erythroid differentiation.^{2,22,52} Erythroid progenitor cells of GATA2 null mice stop differentiating at the rubriblast stage.⁵²

PU.1 is an Ets transcription factor that appears very early in the lineage commitment of HSCs. Its expression is associated with preferential commitment of CMP to granulocyte/monocyte lineage, although an increased ratio of GATA1 to PU.1 expression may cause commitment to erythroid lineage. PU.1 probably does have some direct influence on erythropoiesis, however, by enhancing self-renewal of erythroid progenitors.⁵

Erythroid-Specific Transcription Factors

STAT5 is required for erythropoiesis and is enhanced by EPO and SCF. STAT molecules are mobile cytoplasmic transcription factors that are activated by the JAK family of protein tyrosine kinases.³⁶ STAT molecules are latent but become activated and translocate to the nucleus upon tyrosine phosphorylation. STAT5 binds to specific DNA sequences to up-regulate genes involved in tissue specific functions and regulation of cell growth.⁴⁷ STAT5 knockout mice are severely anemic in utero and their fetal liver cells show increased apoptosis. Some adult STAT5 knockout mice have normal hematocrits, but have ineffective hematopoiesis during erythropoietic stress.⁴⁷ These mice generate increased numbers of proerythroblasts during erythropoietic stress, but have increased apoptosis in the more mature stages.

GATA binding protein 1 (GATA1) is essential for both primitive and definitive erythropoiesis, and plays a key role in the differentiation of rubriblasts to mature red blood cells (RBCs).⁶⁰ GATA1 is responsible for expression of many erythroid-specific or erythroid-predominant genes, including cytoskeletal proteins, the transferrin receptor, EPOR, and the friend of GATA1 transcription factor (FOG-1). GATA1 also maintains expression of Bcl- x_L and EPOR, which promote late erythroid precursor survival and differentiation, while repressing GATA2, a molecule that induces proliferation of earlier stages.

Erythroid Kruppel-like transcription factor (EKLF) is essential for primitive and definitive erythropoiesis and for adult β -hemoglobin production. Its disruption in mice results in accumulation of fetal γ -globin and inability to produce adult β -globin.³⁵ EKLF appears also to regulate expression of other erythroid-specific genes including dematin, cytoskeletal proteins, transcription factors, and heme synthesis proteins.¹⁷

Other Molecules

Bcl- x_L is an anti-apoptotic protein that is associated with the mitochondrial membrane. Bcl- x_L prevents apoptosis of hemoglobinizing rubricytes.³⁹ Mice that have a conditional knock-out of the Bcl- x_L gene have severe anemia and splenomegaly. The bone marrow and spleens of these mice accumulate early rubricytes but are deficient in maturing cells. Bcl- x_L expression is EPO-dependent via STAT5, and is increased during terminal differentiation stages when hemoglobin synthesis is greatest.¹⁵

JAK2 is associated with the EPOR and is responsible for downstream signaling. EPO binding to the EPOR

changes conformation of the receptor and thereby activates JAK2, which causes tyrosine phosphorylation of the cytoplasmic domain, and subsequent recruitment of signaling proteins including STAT5. JAK2 knockout mice die during embryogenesis with severe anemia. The most important function of JAK2 appears to be activation of STAT5.¹⁴

FOG-1 is a nuclear protein that binds to GATA1 and acts as its cofactor in transcription.^{19,25} FOG-1 mediates both transcriptional activation as well as repression (e.g. of GATA2).²⁵ FOG-1 knockout mice die of anemia in utero.⁵³ GATA2 repression is dependent on FOG-1, and thus FOG-1 may facilitate GATA switching, leading to erythroid differentiation.

Negative Regulators of Erythropoiesis

Fas and Fas ligand (FasL) are members of the TNF receptor and TNF superfamilies, respectively, and are expressed as integral membrane proteins. When FasL from one cell interacts with Fas from another cell, clustering of Fas initiates an intracellular caspase cascade and apoptosis. These molecules are co-expressed on a small percentage of immature erythroid cells in murine bone marrow and are responsible for erythroid precursor apoptosis during basal erythropoiesis.³¹ Both of these molecules are down-regulated in response to EPO, allowing erythroid precursor survival and differentiation.

Suppressor of cytokine signaling (SOCS) competes for the binding site of STAT5 on the cytoplasmic tail of the EPOR.⁴⁰ STAT5 can then no longer bind or be activated to produce its downstream effects. SOCS also participates in the degradation of JAK2. SOCS is induced by a number of cytokines, including EPO.⁴³

MICROENVIRONMENT

Erythroblastic islands are one component of the micro-environment (see Chapters 2 and 5) that is essential for erythropoiesis. Erythroblastic islands comprise a central macrophage surrounded by 10–30 erythroid cells from CFU-Es to reticulocytes.²⁸ The macrophage projects cytoplasmic processes to which erythroid cells adhere via erythroblast macrophage protein (EMP), very late antigen 4 (VLA-4), and intercellular adhesion molecule-4 (ICAM-4). These proteins bind to the macrophage receptor proteins EMP, vascular cell adhesion molecule-1 (VCAM-1), and integrins αv and $\alpha 4$, respectively. The macrophage cell surface molecule CD163 also may be important in binding to erythroid cells and in promoting erythropoiesis.¹¹

The central macrophage has a unique phenotype, including a high level of expression of F4/80 and the Forssman glycosphingolipid (a 7 transmembrane spanning receptor), marked adhesiveness, a pronounced ability to endocytose, and inability to generate a respiratory burst. Although erythroblastic islands are critical for erythroblast proliferation and differentiation, macrophage function related to erythropoiesis is incom-

pletely understood. Central macrophages may produce positive and negative regulators of erythropoiesis, supply iron for hemoglobin production, and play a role in nuclear extrusion and phagocytosis of the extruded nucleus.

Molecules Involved in Adhesion

ICAM-4 is a transmembrane protein belonging to the immunoglobulin superfamily of adhesion receptors, and is predominantly expressed on erythroid cells.^{48,49} ICAM-4 mediates binding of rubricytes to rubricytes and rubricytes to macrophages. It is expressed during terminal differentiation of erythroid cells, beginning with the rubriblast. EMP is a 36 kDa transmembrane protein expressed on the surface of erythroid cells and macrophages, and is involved with interactions between erythroid cells and between erythroid cells and macrophages.²⁸ Absence of EMP severely limits the number of erythroblastic islands and results in marked apoptosis of erythroid cells. Very late antigen-4 (VLA-4), also known as $\alpha 4\beta 1$ integrin, is expressed on immature erythroid cells and binds to VCAM-1 on macrophages. Integrin αV is expressed on macrophages and binds to ICAM-4 on immature erythroid cells. Fibronectin is an extracellular matrix glycoprotein that binds to all stages of immature erythroid cells via the VLA-4 and VLA-5 integrins.³⁴

ERYTHROKINETICS

Massive amplification from HSC to mature RBCs is required to create 4×10^{11} mature RBCs from 2×10^4 stem cells.⁶ Erythrokinetics encompasses the rates of proliferation and maturation from HSC to mature RBCs. Long term and short term repopulating cells have been identified in transplantation experiments in mice, and probably represent hematopoietic cells that are less mature than BFU-Es. These cells take months to differentiate into later stages of erythroid cells.¹⁶ BFU-Es take about 10–14 days to develop into rubriblasts in culture, whereas CFU-E maturation takes about 3–5 days. In health, there are approximately 50 rubricytes and 113 reticulocytes for every rubriblast in the bone marrow.¹³ Approximately five mitotic divisions occur over 5 days before a rubriblast loses its nucleus and becomes a reticulocyte. In most species, reticulocytes spend 2–3 days maturing in bone marrow before being released to the circulation.

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Granulopoiesis

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Role of the Microenvironment

Transcription Factor Regulation of Granulopoiesis

Cytokine Regulation of Granulopoiesis

Stem cell factor

Interleukin-3

Granulocyte-macrophage colony stimulating factor

Granulocyte colony stimulating factor

Interleukin-6

Developmental Stages of granulopoiesis

Granulocytes

Neutrophils

Kinetics of neutrophil production

Eosinophils

Basophils

Mast cells

Acronyms and Abbreviations

BaP, basophil progenitor; β c, common beta chain for GP140 type cytokine receptors; BMCP, basophil/mast cell progenitor; C/EBP, CCAAT enhancer binding protein; CD, cluster of differentiation; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; CSF, colony stimulating factor; CXCL12, CXC-chemokine ligand 12; CXCR4, CXCL12 receptor; EoP, eosinophil progenitor; G-CSF, granulocyte colony stimulating factor; GATA-1, GATA binding protein-1; G-CSFR, granulocyte-colony stimulating factor receptor; GFI1, growth factor independent 1; GM-CSF, granulocyte-macrophage colony stimulating factor; GM-CSFR, granulocyte-macrophage colony stimulating factor receptor; GMP, granulocyte/monocyte progenitor; HSC, hematopoietic stem cell; ICAM-1, intercellular adhesion molecule-1; IgE, immunoglobulin E; IL, interleukin; IL-3R, interleukin-3 receptor; IL-5R, interleukin-5 receptor; c-kit, receptor for SCF; M:E, myeloid:erythroid ratio; MCP, mast cell progenitor; MDP, macrophage/dendritic cell progenitor; MEP, megakaryocyte/erythroid progenitor; MPP, multipotential progenitor; mSCF, membrane bound stem cell factor; NeuP, neutrophil progenitor; NGF, nerve growth factor; NK, natural killer; PECAM-1, platelet cell adhesion molecule; SCF, stem cell factor; TGF- β , transforming growth factor- β ; VCAM, vascular cell adhesion molecule; VLA, very late antigen.

In adult mammals, granulopoiesis occurs primarily in the bone marrow with expansion to extramedullary sites in times of increased demand or with bone marrow disease. Most information about development and regulation of the various granulocytic lineages has been obtained from studies using human-derived cells and from rodent models. Conditions for culture of bone marrow influence commitment to and development of various cell lineages from hematopoietic stem cells (see Chapter 133). Culture of bone marrow has been accomplished in most of the common domestic species, and those studies have demonstrated that granulocyte development is similar to rodents and humans.^{19,22,55,57,60}

ROLE OF THE MICROENVIRONMENT

The hematopoietic inductive microenvironment provides the physical structure as well as a chemical milieu permissive for granulopoiesis. The microenvironment

is composed of the extracellular matrix and stromal cells that include mesenchymal stem cells, osteoblasts, endothelial cells, fibroblasts, and adipocytes. Granulopoiesis is regulated by movement of hematopoietic precursor cells through niches in bone marrow in response to a series of factors produced by cells in the microenvironment, interactions with the extracellular matrix, and factors delivered from the blood. Commitment and differentiation are coordinated through changes in expression of cell surface markers, receptors, transcription factors, and signal transduction pathways in the hematopoietic precursors.

In adult mammals, the various granulocytic lineages originate from pleuripotential hematopoietic stem cells (HSCs) within the bone marrow. HSCs reside in close association with osteoblasts along the endosteal border of bone marrow (endosteal niche).^{47,48} Homing of HSCs to the endosteal niche is mediated by chemotactic calcium receptors on HSCs, responding to a calcium concentration gradient generated by osteoclast activity.¹

In addition, HSCs are attracted into the endosteal niche by CXC-chemokine ligand 12 (CXCL12) released by osteoblasts. Chemotaxis is mediated by binding of CXCL12 to the CXCL12 receptor (CXCR4) on HSCs.⁴⁷ The endosteal niche probably is critical for long term survival of HSCs in a quiescent state with a low level of self-renewal.^{47,48,56} Maintenance of HSCs within the endosteal niche appears to be regulated by cell-cell interactions between osteoblasts and HSCs. Osteoblasts express a variety of adherence factors such as VCAM-1 (CD106), N-cadherin, and CD44 that bind respectively to $\alpha 4\beta 1$ integrin (VLA-4 or CD49d/CD29), N-cadherin, and hyaluronic acid on the surface of the HSCs. Osteopontin on osteoblasts binds to both CD44 and $\alpha 1$ integrins on HSCs. Close cell-cell contact permits osteoblast surface ligands to bind to their respective receptors on HSCs, activating intracellular signal transduction within the HSCs. HSCs are maintained in a quiescent state by interactions between membrane bound stem cell factor (mSCF) on osteoblasts with the c-kit tyrosine kinase on HSCs and by angiopoietin-1 binding to Tie-2 tyrosine kinase.⁵⁶ Apoptosis of HSCs is inhibited by mSCF/KIT and Flt ligand/Flt-3 tyrosine kinase initiation of the Mcl-1 pathway.²⁶ Self-renewal is promoted by Jagged-1/Notch-1 and m-SCF/c-kit signaling.

Proliferation, mobilization, and commitment of HSCs to granulocytic differentiation occur in proposed vascular niches along the vascular sinuses of the central bone marrow.³² Homing of HSCs and hematopoietic progenitor cells to the vascular niche occurs in response to CXCL12 secreted by endothelial cells. Retention in vascular areas of the bone marrow results from binding of HSCs and progenitors to PECAM-1 (CD31), VCAM-1 (CD106), P-selectin, and E-selectin found on the surface of endothelial cells. The vascular niche may facilitate differentiation of HSCs to CD34+ multipotential progenitors (MPPs) that have multilineage potential but have lost the ability of self-renewal. Subsequent to this stage, progenitors for lymphocyte and myeloid lineages commit to either common lymphoid progenitors (CLPs) or common myeloid progenitors (CMPs).

It is currently proposed that CMPs give rise to several lineages including granulocyte/monocyte progenitors (GMPs), megakaryocyte/erythroid progenitors (MEPs), and macrophage/dendritic cell progenitors (MDPs).⁴² Granulocytic lineages differentiate from GMPs to produce neutrophil progenitors (NeuPs), eosinophil progenitors (EoPs), and basophil/mast cell progenitors (BMCPs).² BMCPs subsequently give rise to basophil progenitors (BaPs), which complete their maturation in bone marrow, and mast cell progenitors (MCPs), which circulate and undergo maturation in tissues such as gastrointestinal mucosa, skin, lung, and heart. An alternate proposed origin of MCP is directly from MPP.⁸

Although it is conceptually helpful to think of progenitor development in a linear model, lineage differentiation may vary with species or may be more plastic than a linear model implies. This is evident in leukemic cell lines that express markers for more than one hematopoietic lineage and by the capacity to change differen-

tiation of cultured cells by altering patterns of transcription factor expression.

TRANSCRIPTION FACTOR REGULATION OF GRANULOPOIESIS

Commitment, differentiation, and maturation of granulocytic progenitors results from a coordinated program of transcription factor expression that activates genes required for differentiation and maturation of a specific cell type (Fig. 7.1).^{17,38,42,50,54} Suppression of genetic programs required for differentiation along other cell lines is equally important in lineage determination. The coordinated hierarchy of transcription factor activation and suppression results in expression of receptors for colony stimulating factors (CSF) on the surface of hematopoietic progenitors, affects the timing of adhesion molecule expression, and activates genetic programs that allow transition to committed lineages and functional maturation within a lineage. Shifts in the balance of transcription factors may allow transition of progenitors to alternate cell lineages.

Differentiation of MPPs to CMPs requires activation of PU.1, a member of the Ets transcription factor family.^{17,54} PU.1 in conjunction with expression of CCAAT/enhancer binding protein- α (C/EBP- α), a basic-region leucine zipper transcription factor, promotes transition to GMPs. Continued presence of C/EBP- α and a lower level of expression of PU.1 results in formation of early neutrophil precursors. This is in contrast to those GMPs that maintain high levels of PU.1 but no longer express C/EBP- α , which results in commitment to monocyte production. Maturation of neutrophils beyond the promyelocyte stage is dependent on increasing expression of PU.1 as well as C/EBP- ϵ , another C/EBP family member, and growth factor independent-1 (GFI-1). Production of EoP results from expression of GATA-1 (zinc finger family transcription factors) and continued high levels of C/EBP- α , whereas BMCP formation requires GATA-2 expression but inactivation of C/EBP- α .^{2,20,21} For BMCPs to commit to production of basophils, there must be reactivation of C/EBP- α to produce BaPs, whereas continued down-regulation of C/EBP- α is required for differentiation to MCPs.

CYTOKINE REGULATION OF GRANULOPOIESIS

Granulopoiesis is regulated by cytokines that include stem cell factor (SCF) and CSFs.^{4,6} Expression of receptors for these cytokines on the surface of hematopoietic cells is controlled by the activity of transcription factors so that sensitivity to individual cytokines coordinates with and facilitates proliferation and differentiation of the various cell lineages. Most hematopoietic cytokines are constitutively produced at low levels by a variety of cell types to maintain steady state granulopoiesis but increase in response to inflammation.

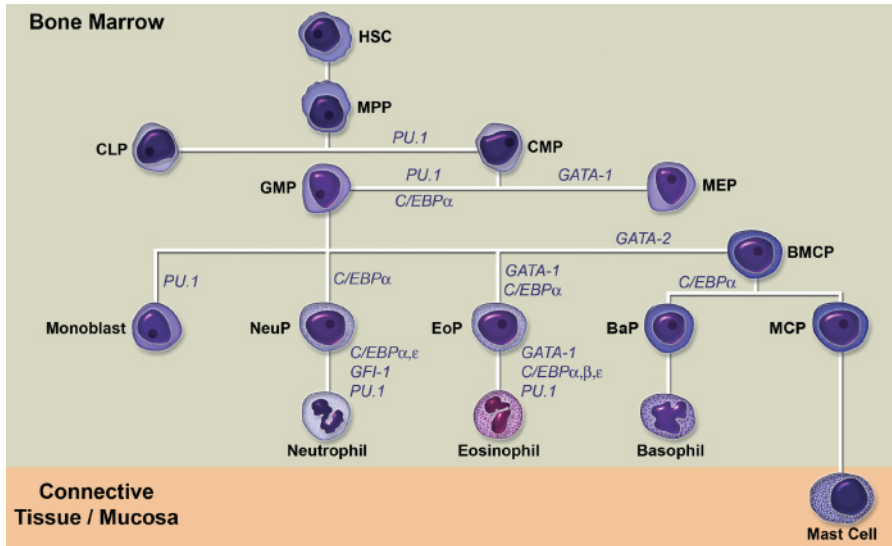


FIGURE 7.1 Proposed scheme for granulopoiesis. Transcriptional regulation of granulopoiesis is indicated. MCP also may arise directly from MPP. (BaP, basophil progenitor; BMCP, basophil/mast cell progenitor; C/EBP, CCAAT enhancer binding protein; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; EoP, eosinophil progenitor; GMP, granulocyte/monocyte progenitor; HSC, hematopoietic stem cell; MCP, mast cell progenitor; MEP, megakaryocyte/erythroid progenitor; NeuP, neutrophil progenitor). (Illustrated by Tim Vojt, Biomedical Media, The Ohio State University College of Veterinary Medicine.)

Stem Cell Factor

Stem cell factor (SCF; kit ligand, Steel factor, mast cell growth factor) acts on some of the earliest hematopoietic progenitors and is required for survival and self-renewal of HSCs (see Chapter 3).^{6,46} Stem cell factor is produced as either a membrane-bound (mSCF) or soluble form. mSCF appears to be critical for effective hematopoiesis as soluble SCF is unable to compensate for lack of production of the membrane-bound form in genetically engineered mice.⁶ mSCF is found on osteoblasts, endothelial cells, and fibroblasts in the bone marrow, and plays a role in establishing progenitors in their appropriate bone marrow niches. SCF acts to both inhibit apoptosis as well as promote entry of cells into the S phase of the cell cycle. SCF also potentiates the effects of other cytokines and CSF to promote proliferation, especially of early committed progenitors. c-kit (CD117), a member of the type III receptor tyrosine kinase family, is the receptor for SCF. Binding of SCF to c-kit results in homodimerization and autophosphorylation of the receptor. Expression of c-kit varies depending on the stage of hematopoietic cell development.²⁵ c-kit expression is present at low levels on quiescent HSCs and increases to its highest levels on early, actively proliferating granulocytic progenitors. With the exception of mast cells, c-kit expression declines with further maturation of the granulocytic lineages.

Interleukin-3

Interleukin-3 (IL-3), a member of the short chain α -helical bundle type 1 cytokine family, acts on some of the earliest hematopoietic progenitors.⁴ It is primarily produced by activated T-lymphocytes; however, other cell types such as bone marrow stromal cells, natural killer (NK) cells, eosinophils, and mast cells also can produce IL-3. Its effects are mediated through binding to a specific receptor (IL-3R). IL-3R is a heterodimer consisting of α and β chains. The α chain (IL-3R α or

CD123) conveys ligand specificity. The common β chain (β c or CD131), upon dimerizing with the α chain–ligand complex, initiates signal transduction. Transcription of the IL-3R α is controlled by transcription factors PU.1, GATA-1, and C/EBP α , whereas constitutive expression of the β c appears to be controlled by PU.1. IL-3R is found on early progenitors such as CD34⁺ HSCs, MPPs, and CMPs. The primary effects of IL-3 are to promote proliferation, differentiation, and survival of the most primitive precursors by shortening the time in the G₀ stage of the cell cycle. Although IL-3R may be found on more mature granulocyte precursors, the growth-promoting effects of IL-3 continue to be important for eosinophils and basophils.

Granulocyte-Macrophage Colony Stimulating Factor

Granulocyte-macrophage CSF (GM-CSF), a member of the short chain α -helical bundle type 1 cytokine family, is produced by a variety of cells including macrophages, T and B cells, bone marrow stromal cells, osteoblasts, fibroblasts, endothelial cells, neutrophils, eosinophils, and mast cells.⁴ The receptor for GM-CSF (GM-CSFR) is also a member of the GP140 cytokine receptor superfamily. GM-CSFR is a heterodimer that is structurally related to and shares the same β c with the IL-3R but has a unique α chain (GM-CSFR α or CD116) that confers ligand specificity. Like IL-3R, transcription of the GM-CSFR α is controlled by PU.1, GATA-1, and C/EBP. GM-CSFR is expressed as progenitor cells commit to granulocytic and monocytic differentiation. Binding of GM-CSF to its receptor promotes progenitor survival by inhibiting apoptosis. GM-CSF promotes proliferation and maturation of granulocytes and macrophages.

Granulocyte Colony Stimulating Factor

Granulocyte CSF (G-CSF) is produced primarily by macrophages and monocytes. However, it also

may be produced by fibroblasts, endothelial cells, T-lymphocytes, and bone marrow stromal cells.⁴ The receptor for G-CSF (G-CSFR) is a member of the GP130 cytokine receptor superfamily and forms a homodimer or homo-oligomer upon ligand binding. G-CSFRs are expressed in highest concentrations by neutrophilic progenitors through mature neutrophils. Sensitivity to G-CSF increases with neutrophil maturation and is related to increasing numbers of G-CSFR.³⁸ The effects of G-CSF are wide ranging and include stimulation of survival, proliferation, and terminal differentiation of neutrophils. G-CSF is constitutively produced at low levels to support basal neutrophil production. Concentrations of G-CSF increase markedly in response to endotoxin and inflammatory mediators, thus driving increased neutrophil release and production during an inflammatory response.³⁸ G-CSF also shortens maturation time of neutrophilic precursors, decreases marrow transit time, stimulates release of neutrophils from bone marrow, and prevents apoptosis. High concentrations of G-CSF result in release of HSCs into the circulation. This technique has been used to harvest circulating HSCs for bone marrow transplantation procedures in humans. Release of HSC in response to G-CSF appears to be mediated in part by elaboration of neutrophil enzymes, such as neutrophil elastase, cathepsin G, and matrix metalloproteinase-9 within the bone marrow microenvironment.³² These enzymes cleave adhesion molecules on HSCs and osteoblasts, thereby releasing HSCs.

Interleukin-6

IL-6 is a member of the GP130 cytokine family, and receptors for IL-6 are found on some CD34+ hematopoietic precursors.²⁴ IL-6 can stimulate granulopoiesis via binding to membrane bound IL-6 receptor. GP130 signaling can also be initiated through the mechanism of "transsignaling" wherein IL-6 is bound to a soluble form of its receptor and this complex heterodimerizes with GP130 to activate signaling. This latter mechanism appears to be important in granulopoiesis.^{23,39} IL-6 can act synergistically with SCF to enhance granulopoiesis in culture and can partially compensate for lack of G-CSF in knockout mice.^{33,39} IL-6 can act alone or synergistically with G-CSF to mediate bone marrow granulocytic hyperplasia during times when there is increased demand for neutrophils.⁵³

DEVELOPMENTAL STAGES OF GRANULOPOIESIS

Committed neutrophil, eosinophil, and basophil precursors undergo a similar maturation sequence (Fig. 7.2). The myeloblast is the first member of granulocytic lineage that is recognizable by light microscopy. The myeloblast is characterized by finely stippled chromatin, one to multiple nucleoli, and moderately basophilic cytoplasm. Initially, the cytoplasm lacks granules (Type I myeloblast), but small numbers of primary or

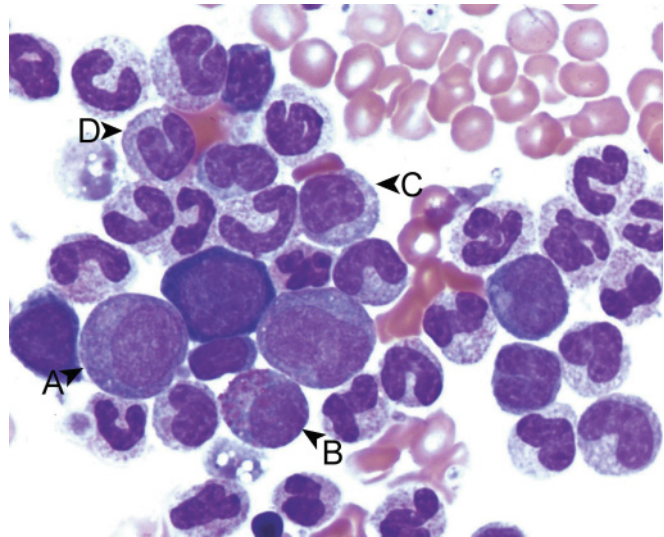


FIGURE 7.2 Granulocytic precursors in canine bone marrow. (A) promyelocyte, (B) eosinophilic myelocyte, (C) metamyelocyte, (D) band neutrophil. Wright Giemsa stain; original magnification 1000 \times .

azurophilic granules begin to appear as the myeloblast matures (Type II myeloblast). The promyelocyte (progranulocyte) is larger than the myeloblast and is characterized by increasing numbers of primary granules and decreasing cytoplasmic basophilia. A Golgi area may be apparent and is free of granules. The chromatin pattern remains finely stippled and nucleoli may or may not be present. As promyelocytes mature, primary granules become less apparent by light microscopy and secondary granules appear. The myelocyte is smaller than the promyelocyte and is characterized by a round to slightly oval nucleus with partially condensed chromatin and lightly basophilic cytoplasm containing secondary granules. The myelocyte is the stage that commitment to a specific granulocytic line can most easily be discerned by light microscopy due to the presence of lineage specific secondary granules. The myelocyte is considered the last stage at which cellular division occurs. For each myeloblast, there is the potential to produce 16–32 offspring.

Metamyelocytes and band granulocytes are no longer capable of cell division, but undergo nuclear maturation characterized by increasing condensation of chromatin and progressive segmentation of the nucleus. The metamyelocyte has a bean-shaped to elongated nucleus whereas the band has a U- or an S-shaped nucleus with smooth, parallel sides. As these cells mature from metamyelocyte to bands, the cytoplasm becomes less basophilic and granule contents mature. Mature granulocytes have condensed chromatin and a segmented nucleus with multiple lobes separated by thin strand-like areas. The degree of segmentation varies with the granulocyte type and species. In general, segmented neutrophils have 3–5 lobes whereas eosinophils may be bilobed. Mature basophil nuclei may appear monocytoïd to bilobed.

GRANULOCYTES

Neutrophils

Differentiation from CMPs to GMPs is stimulated by IL-3, GM-CSF, and IL-6. Commitment of GMPs to neutrophil production and proliferation, and maturation of myeloblasts to mature polymorphonuclear neutrophils is primarily driven by G-CSF. Although IL-6 can stimulate granulopoiesis and partially compensate for a lack of G-CSF in knockout mice, G-CSF is required for functional maturation of neutrophils by increasing adhesion factors, chemotactic activity, phagocytosis, and production of bactericidal factors.³⁸

Development of myeloblasts to segmented neutrophils is associated with changes in cell surface expression of adhesion molecules and receptors.¹⁴ In humans, maturation from myeloblasts to segmented neutrophils is accompanied by decreased surface expression of adhesion molecules that regulate retention in bone marrow such as CD29, CD31 (PECAM-1), CD33, CD49d (binds to VCAM), and CD49e (binds to fibronectin). There is increased expression of other adhesion molecules associated with neutrophil rolling and adherence (e.g. CD11b, CD11c) as cells mature. There is decreased expression of CD54 (ICAM-1) during neutrophilic maturation, but its expression may be reactivated when neutrophils are exposed to high concentrations of G-CSF during inflammation. Receptors for immunoglobulin (CD16 and CD32) and receptors for or modifiers of complement activity (CD11b, CD11c, CD35, and CD55) tend to increase with maturation.

Kinetics of Neutrophil Production

Neutrophil development can be divided conceptually into proliferation (mitotic), maturation, and storage compartments. The proliferation pool encompasses those precursors that are capable of cell division, including myeloblasts, promyelocytes, and myelocytes. These precursors constitute approximately 10–30% of bone marrow granulocytes in most species. The maturation and storage compartments comprise 65–90% of the granulocytes in the marrow. The maturation compartment consists of metamyelocytes and band granulocytes that are no longer capable of mitosis but are still undergoing nuclear and cytoplasmic maturation. The storage compartment comprises those bands and segmented neutrophils that are readily released into circulation.

Under basal conditions, bone marrow transit time for neutrophils (the time to go from myeloblast to segmented neutrophil) is approximately 6 days in dogs, cats, and horses.^{5,7,13,40} Production time for neutrophils in calves is 6 days, but neutrophils are held in the bone marrow for one additional day before release.^{51,52} Delivery of neutrophils to blood is related to the rapidity with which neutrophils are depleted from the circulation.⁵¹ Although the steady state bone marrow reserve of neutrophils is similar in dogs and calves, the proliferative capacity of bone marrow in calves appears to be

less than the dog. This is reflected in the lower M:E ratio in cattle. If neutrophils are depleted from the circulation, dogs react with a leukocytosis and bone marrow hyperplasia characterized by a shortening of production time from 6 to 2–3 days.^{5,51} Calves also deliver existing neutrophils and bands from the maturation and storage pools to the blood in response to increased movement of neutrophils out of the circulation. However, unlike in other species, there is no apparent shortening of transit time as part of the bone marrow response in calves.^{51,52} The mechanism underlying the failure of cattle to increase granulopoiesis and shorten bone marrow transit time is uncertain. Increased demand for neutrophils results in release of inflammatory cytokines and increased production of G-CSF.³⁸ G-CSF is a key mediator of neutrophil mobilization from bone marrow reserves, stimulation of granulopoiesis, and functional maturation of neutrophil precursors. Dogs, cats, horses, and cattle all show a robust response to administration of G-CSF characterized by neutrophilia and granulocytic hyperplasia in the bone marrow.^{9,10,18,35,37,44}

Eosinophils

EoPs originate from GMPs, the same progenitors that give rise to neutrophils. However, commitment to eosinophil development occurs in response to a different pattern of transcription factors and cytokines.^{20,58} The main cytokines driving eosinophil production are IL-3, GM-CSF, and IL-5. Eosinophils appear to lack receptors for G-CSF.³⁶ IL-3 and GM-CSF play a role in the early steps of proliferation and commitment of the eosinophil progenitor pool, whereas IL-5 is required for proliferation, terminal differentiation, and functional maturation of eosinophils.⁴³ The receptor for IL-5 (IL-5R) shares the same signal transducing β c with IL-3 and GM-CSF but has a unique α -chain (IL-5R α or CD125) to permit specific binding of IL-5. Thus, eosinophils differ from neutrophils in that β c signal transduction is required through all stages of eosinophil production and maturation. Th2 T-helper cells are the primary source of IL-5. However, IL-5 can be produced by other cells such as mast cells, eosinophils, Tc2 cells, $\gamma\delta$ T cells, NK cells, and epithelial cells.⁴⁹ In addition to its role in proliferation and differentiation of eosinophils, IL-5 prevents apoptosis, stimulates release of eosinophils from the bone marrow, promotes adhesion of eosinophils to endothelial cells, and activates eosinophil effector function. Overproduction of IL-5 is associated with peripheral eosinophilia in transgenic mice and is observed in various naturally-occurring and experimentally-induced hypereosinophilic conditions. Interestingly, IL-5 knockout mice have normal basal numbers of eosinophils in their bone marrow but fail to develop tissue or blood eosinophilia when stimulated by parasite infection or inhalant allergen.^{16,29} This suggests that IL-5 is necessary for an expanded eosinophilic response to inflammatory stimuli.

Bone marrow production time from myeloblast to mature eosinophils takes 2–6 days.⁵⁹ Secondary or

specific granules begin to form at the late promyelocyte stage and granule contents continue to mature through the segmented stage. Production of specific granule proteins occurs in response to genetic pathways activated by the transcription factors described above. For example, production of major basic protein is controlled by C/EBP ϵ , GATA-1 and PU.1.²⁰

Basophils

Committed basophil progenitors (BaPs) originate from CD34+ myeloid progenitors in the bone marrow. However, the sequence of intermediaries between MPPs and BaPs remains unclear. Studies of bone marrow cells from humans suggest that basophils and eosinophils share a bipotential progenitor because eosinophils and basophils develop within the same colony under some experimental culture conditions.^{12,31} This may reflect the fact that the proliferation and differentiation of eosinophils and basophils are stimulated by many of the same cytokines such as IL-3 and GM-CSF.³ Studies using bone marrow cells from mice suggest that basophils share a common progenitor with mast cells (BMCP) and originate from GMPs.^{2,21} Subsequent differentiation to either BaPs or MCPs is regulated by sequential expression of transcription factors. Up-regulation of GATA-2 in conjunction with down-regulation of C/EBP α induces differentiation to BMCPs capable of differentiating into either basophils or mast cells. Subsequent reactivation of C/EBP α results in committed BaPs and production of basophils.^{2,21} Maturation of BaPs occurs in bone marrow, and transit time from myeloblasts to mature basophils takes 2–3 days.⁴⁵ Basophil precursors require β c signal transduction initiated by IL-3 and GM-CSF for proliferation and differentiation. IL-5, nerve growth factor (NGF), and transforming growth factor- β (TGF- β) also act as growth factors.¹⁵ However, unlike other granulocytes, a lineage specific factor (a specific basophilopoietin) has not been identified. It has been speculated that differentiation to basophil lineage is a default pathway, which may contribute to the conflicting findings as to their origin.³ Although IL-3 appears to be the main growth factor promoting differentiation and maturation of basophil precursors, it is not absolutely required for production of mature basophils in steady state conditions as evidenced by studies with IL-3 deficient mice.³⁰ However, IL-3 is needed for accelerated production leading to basophilia in response to parasites.³⁰ Both IL-3 and GM-CSF increase effector function of basophils by increasing histamine production through induction of histidine decarboxylase, increasing regulatory cytokine production (such as IL-4 and IL-13), and enhancing migration of mature basophils into tissues.

Mast Cells

Mast cell progenitors originate from CD34+ hematopoietic progenitors in the bone marrow. Early developmental pathways remain unclear and studies suggest

various schemes, including common BMCPs descended from GMPs or by direct derivation from MPPs.^{2,8,21} Unlike granulocytic lineages, committed MCPs exit the bone marrow and undergo proliferation, differentiation, and maturation in mucosal and connective tissues. At the time of migration, these MCPs lack mature granules and the high affinity receptor for the Fc portion of IgE.⁴¹

IL-3 and SCF are the primary growth factors for mast cell development.^{27,28} Unlike the other granulocytic lineages, MCPs maintain high levels of c-kit, the receptor for SCF.³⁴ Stimulation of c-kit supports proliferation and differentiation of mast cells, and its effects may be potentiated by co-incubation with IL-3. In addition, SCF binding to KIT inhibits apoptosis and prolongs survival of mast cells. SCF is a chemotactic agent for immature and mature mast cells and helps localize mast cells to tissues.¹¹

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Monocytes and Dendritic Cell Production and Distribution

TRACEY L. PAPENFUSS

Monocyte and Dendritic Cell Production
Transcriptional and Cytokine Regulation
 Transcriptional Regulation
 Cytokine Regulation
Effects of Altering Key Cytokines
Generation and Expansion

Circulation, Tissue Distribution, and Function $\beta\beta$
 Monocytes
 Macrophages
 Dendritic Cells
Differentiating Monocytes, Macrophages, and
 Dendritic Cells
Summary

Acronyms and Abbreviations

C/EBP- α , CCAAT/enhancer binding protein-alpha; CD, cluster of differentiation; CFU-GM, colony forming unit granulocyte macrophage; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; cDCs (conventional DCs); DCs, dendritic cells; DNA, deoxyribonucleic acid; FIV, feline immunodeficiency virus; Flt3L, Flt3-ligand; GM-CSF, granulocyte macrophage colony stimulating factor; GMP, granulocyte/monocyte progenitors; HSC, hematopoietic stem cells; iNOS, inducible nitric oxide synthase; IDO, indoleamine-2,3-dioxygenase; IL, interleukin; IRF8, interferon- γ -responsive transcription factor; KO, knock-out; LC, Langerhans cells; M-CSF, macrophage colony stimulating factor; MDP, macrophage/dendritic cell progenitors; MHC, major histocompatibility antigen; MEP, megakaryocyte/erythroid progenitors; MPP, multipotential progenitors; MPS, mononuclear phagocytic system; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; pDCs, plasmacytoid DCs; SCF, stem cell factor; TGF- β , transforming growth factor-beta; TTP DCs, tumor necrosis factor- α (TNF- α) inducible nitric oxide synthase (iNOS)-producing DCs; TLR, Toll-like receptor; TNF- α , tumor necrosis factor-alpha; Treg, regulatory T (cell); V-RelB, reticuloendotheliosis viral oncogene homolog B SCF.

The mononuclear phagocytic system (MPS) is a term that is commonly used to describe monocytes, macrophages and their progenitor cells. Although a somewhat imprecise term, the MPS is useful in describing the heterogeneity yet inter-relatedness of these cells. Dendritic cells (DCs) are bone marrow-derived cell that are not traditionally included in the MPS but can arise from common myeloid precursor cells or monocytes and share characteristics with monocytes and macrophages. The term histiocyte is relatively imprecise but is a useful term to describe cells in cytologic or histopathologic specimens considered to be of MPS origin, but whose precise identity is unknown. In this chapter, DCs, monocytes, and macrophages will be discussed together despite the fact the DCs are distinct in many ways. Cells of the MPS play an important role in phagocytosis and killing of infectious agents, diges-

tion of cellular debris, and are key players in both innate and adaptive immune responses.

This chapter will discuss the formation of monocytes, macrophages, and DCs, and contrast differences in form and function between these cells.

MONOCYTE AND DENDRITIC CELL PRODUCTION

The hematopoietic stem cell (HSC) is a pluripotent progenitor cell that gives rise to common lymphoid and common myeloid progenitors (CLPs and CMPs, respectively). The CMP gives rise to numerous progenitors including granulocyte/monocyte progenitors (GMPs), macrophage/dendritic cell progenitors (MDPs) and megakaryocyte/erythroid progenitors (MEPs), while a

subset of DCs arise from CLPs.¹¹ The fact that CLPs and CMPs can give rise to DCs led to original classification of DCs as lymphoid or myeloid based on cluster of differentiation (CD)8 α expression with proposed distinct functions of these subsets. More recent work indicates that these subsets can arise from either CLP or CMP and are currently referred to as conventional DCs (cDCs). Although plasmacytoid DCs (pDCs) also can arise from either CLP or CMP, their unique function sets them apart from cDCs. Through an orchestrated sequence of transcriptional events and local cytokine microenvironmental stimuli, GMPs, MDPs and CLPs give rise to monocytes, macrophages and DCs.

TRANSCRIPTIONAL AND CYTOKINE REGULATION

When discussing the origin and development of monocytes, macrophages, and DCs, it is important to understand the role and diversity of stem cells. Our understanding of hematopoiesis has arisen primarily from human and mouse studies and through the use of genetically engineered (i.e. knock-out) mice (GEMs). Although such GEMs are powerful tools, they have limitations, particularly when the altered gene plays a critical or multi-factorial role in hematopoiesis or embryological development. However, even with such limitations, GEMs are powerful tools that have been instrumental in unraveling key events, transcriptional regulators, and cytokines involved in hematopoiesis and the development and regulation of monocytes, macrophages, and DCs.

Transcriptional Regulation

PU.1 is an early transcription factor important in differentiation of CMPs and CLPs from MPP whose presence allows differentiation along the myeloid lineage and whose absence promotes erythroid development.¹¹ The coordinated expression of transcription factors PU.1, CCAAT/enhancer binding protein- α (C/EBP- α) and interferon- γ -responsive transcription factor (IRF8) influence development of granulocytic versus monocytic/macrophage lineages.¹¹ GMPs under the influence of decreasing PU.1 levels and increasing C/EBP- α will form granulocytes. Conversely, high PU.1, lower C/EBP- α and increased expression of IRF8 will promote generation of monocytes and macrophages, with IRF8 acting through unknown influences on M-CSF signaling.¹¹ Due to the CMP and CLP origin of some DCs, Ikaros, an important transcription factor involved in lymphopoiesis, plays an essential role in development and differentiation of DCs such as lymphoid DCs.² DC subset lineage commitment may be influenced by additional transcription factors such as V-rel reticuloendotheliosis viral oncogene homolog B (RelB), a member of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) family which is expressed in higher amounts in CD8 α - cDCs, and inhibitor of dif-

ferentiation 2 (Id2) which is upregulated during DC development and required for the development of cDCs and Langerhans cells (LCs).

Cytokine Regulation

Numerous cytokines play an important role in shaping the microenvironment and promoting differentiation of specific cell populations. Under the influence of interleukin-1 (IL-1), IL-3, IL-6 and stem cell factor (SCF) Multipotential progenitors (MPPs) will form CFU-GM/CMP from which either the granulocytic or monocytic lineage will develop.^{3,15} Monocyte production is stimulated by SCF, macrophage-colony stimulating factor (M-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), IL-1, IL-3, and IL-6 and increased PU.1 expression.³ Monocytes arise through sequential differentiation of CFU-GM to monoblasts, promonocytes, and finally to monocytes (Fig. 8.1). M-CSF is a critical cytokine involved in the formation of macrophages and monocyte-derived cells with macrophage functions such as osteoclasts. Subsequent maturation and function of tissue macrophages is determined by the local tissue microenvironment. Flt3-ligand (Flt3L) is the most important cytokine involved in generating all DC subsets and plays an important role in maintaining steady-state levels of DCs. GM-CSF promotes development of inflammatory DCs and induces differentiation of monocytes and bone marrow cultures to DCs. Similar to macrophages, local tissue environment alters DC phenotype and function. One such example is the critical role played by TGF- β in generating LC.¹

Effects of Altering Key Cytokines

Our understanding of the importance of various cytokines in the generation of monocytes, macrophages and DCs has arisen primarily by GEM knock-out (KO) mice.

The absence of M-CSF or its receptor results in decreased numbers of monocytes and certain macrophage subsets. M-CSF does not appear to be critical for steady-state DC production because DC numbers, ratios, and function appear to not be affected in these KO mice. Surprisingly, GM-CSF does not appear to be essential during normal hematopoiesis because KO mice have normal hematopoiesis. GM-CSF does appear to play a primary role in generating and maintaining DCs in inflammatory states and can act synergistically with Flt3L.^{13,17} Flt3L is the major cytokine driving steady-state DC production in vivo because mice with knock out of the Flt3L or Flt3L signaling pathways have a significant deficiency in DCs.⁸ However, these mice have normal monocyte development and GM-CSF-mediated DC development. Flt3L administration in vivo increases DCs and appears to act synergistically with GM-CSF to expand DCs in vivo while GM-CSF alone has little effect on DC numbers in vivo.⁴ TGF- β plays an essential role in the generation of skin DCs (i.e. LC) because mice that lack TGF- β are deficient in LC.⁹

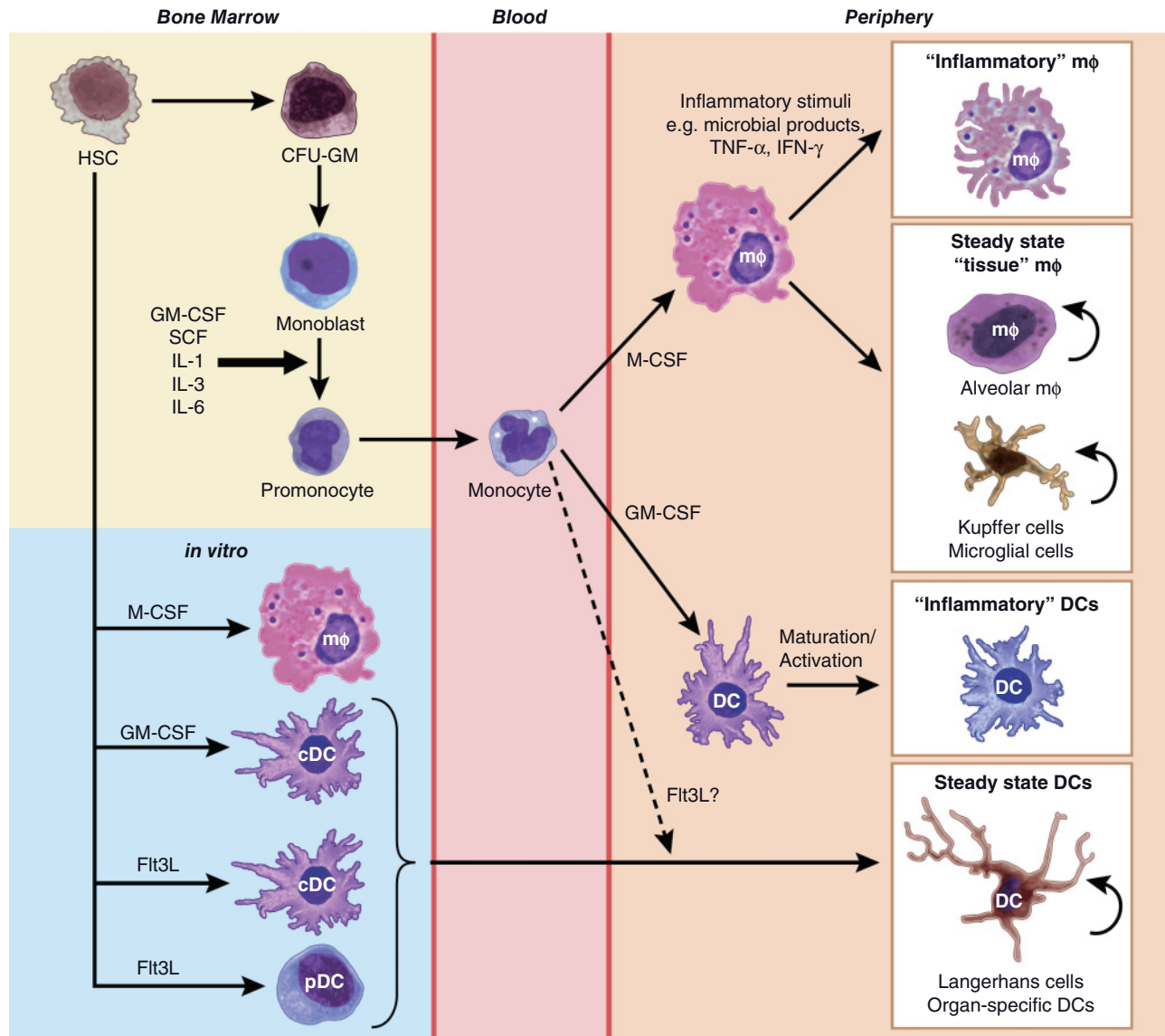


FIGURE 8.1 Location and developmental pathways of monocytes, macrophages (mΦs) and dendritic cells (DCs). Hematopoietic stem cells (HSCs) give rise to CFU-GM which can then give rise to monocytic (and granulocytic) precursors. Monocytes circulate in blood and can develop into either macrophages or DCs. M-CSF promotes development of mΦs from monocytes. GM-CSF promotes development of DCs. Inflammatory stimuli and other mΦ factors can give rise to “inflammatory” mΦ and DC populations. In steady state, tissue mΦs and DCs are replenished by either circulating monocytes or self-renewal. In vitro, HSCs give rise to mΦs when cultured with M-CSF, conventional DCs (cDCs) when cultured with GM-CSF, and cDCs and plasmacytoid DCs (pDCs) when cultured with Flt3L. Flt3L acts on bone marrow progenitors to generate both cDCs and pDCs that represent “steady state” DCs. GM-CSF acts on monocytes to generate DCs with an “inflammatory” phenotype.

Generation and Expansion

Monocyte precursors and monocytes can give rise to macrophages or DCs in vitro depending on culture conditions, cytokines, and growth factors. M-CSF is a primary cytokine used to generate macrophages from monocyte precursors or bone marrow mononuclear cells in culture.¹⁶ GM-CSF is the primary growth factor used to generate DCs in vitro, whereas Flt3L has been used in vitro and in vivo to generate DCs.¹⁷ Flt3L is capable of generating all DC subsets in vitro including both cDCs and pDCs, while GM-CSF prevents the

generation of pDCs. In vivo, DC expansion is effectively accomplished by administration of Flt3L.⁴ GM-CSF does not expand DCs in vivo but does appear to amplify in vivo expansion if administered with Flt3L.⁴ Flt3L binds Flt3 expressed on early bone marrow progenitors, which stimulates proliferation, differentiation, mobilization, and survival of these cells. Although Flt3L can generate a monocytopoiesis and is important in producing steady-state DCs, Flt3L alone is not capable of producing monocyte-derived DCs, probably due to low Flt3 expression on monocytes.

CIRCULATION, TISSUE DISTRIBUTION, AND FUNCTION

Macrophages

Macrophages are rarely present in blood. In tissues, multinucleated giant cells, syncytial cells, and epithelioid macrophages may develop in chronic inflammation or in response to specific infectious agents (see Chapter 45). Tissue-specific macrophages have been recognized for decades and have various names depending on location. Two such examples include Kupffer cells in the liver and microglial cells in the central nervous system. Such tissue macrophages often establish in specific organs during embryogenesis and maintain steady-state levels primarily by local replication with only a small contribution from monocytes during homeostasis. The majority of inflammatory tissue macrophages arise from monocytes recruited from blood. Regardless of location, tissue macrophages have similar functions which include surveillance, removal of dead cells and cellular debris, defense against pathogens, and promotion of wound healing, tissue remodeling, and repair. Tissue macrophages have a life span of years and can divide, but often do so at a very low rate.

Dendritic Cells

DCs can arise from HSCs, before an absolute commitment of HSCs to CMPs or CLPs. This fact makes DCs unique as cells in the MPS and challenges a linear model of DC genesis. Thus, unlike the lineage-specific model of development where CMPs differentiate into monocytes which give rise to macrophages, a graded commitment model of DC development has been proposed. In this model, early precursors (HSC and CMP) are more likely to generate both pDCs and cDCs while more differentiated stages of DCs (e.g. pro- or pre-DCs) will more likely give rise to cDCs including the CD8⁺ and CD8⁻DC subtypes.⁹ Thus, DC development results from a combination of transcription factors and external cytokine signals influencing pDC and cDC development from both the myeloid and lymphoid pathways.

Similar to macrophages, DCs are not typically found in the circulation. However, circulating pre-DCs have been demonstrated in both humans and mice and arise from bone marrow rather than recirculation of DCs.⁹ Once mature, DCs can be classified as migratory, resident, circulating, or inflammatory and be inactive or activated in each of these capacities.⁹

Differentiation of monocytes to DCs is more rapid during inflammation (18 h) than generation time of DCs *in vitro*. The half-life for activated DCs in inflammation is 3–5 days. Approximately 5–10% of resting or tissue DCs are in cell cycle at any given time, and the relative contribution of monocytes and bone marrow precursors to normal DC replenishment, turnover, and recruitment is less well characterized than for tissue macrophages.⁷

Dendritic cells have a wide tissue distribution and are located in all organs throughout the body. In such sites, DCs are positioned to serve as sentinels of the immune response and can initiate and direct subsequent immune responses. Many cells previously identified as tissue macrophages are now recognized as DCs or mixed populations of DCs and macrophages. LC are DCs in the skin and, together with dermal macrophages, are important in cutaneous immune responses.¹⁰ DCs and macrophages in the lamina propria of the gut play distinct roles in directing immune responses that may influence development of autoimmune and inflammatory diseases.

Cell surface markers are commonly used to identify DCs and their subsets. In general, CD8 α ⁺ cDCs are thought to play a role in maintaining homeostasis and tolerance to self and are commonly found in T cell-rich areas of the spleen and lymph nodes. In contrast, CD8 α ⁻ cDCs comprise nearly 80% of resident DCs in the spleen, are a minor population in lymph nodes, and tend to be found in the marginal zones with subsequent redistribution to T cell areas upon stimulation through toll like receptors (TLRs).⁹ These CD8 α ⁻ cDCs are thought to be the primary DCs that initiate immune responses.

Activation of immature DCs results in migration to lymph nodes, up-regulation of surface expression of major histocompatibility antigens (MHC) Class II and co-stimulatory markers, and production of cytokines (e.g. IL-1, IL-6, IL-10, IL-12, IL-23, TGF- β , TNF- α , type I interferons), chemokines, and other soluble factors (e.g. indoleamine-2,3-dioxygenase IDO).¹² DCs then interact with numerous other cells including both immune and non-immune (e.g. endothelial cells) to initiate and direct an immune response (see Chapter 51).

The microenvironment plays an important role in determining whether DCs become immunogenic or regulatory. An inflammatory stimulus causes DCs to mature from processing antigen to actively presenting antigen and initiating an immune response. Immunogenic DCs up-regulate MHC Class II and co-stimulatory molecules, and migrate to local lymph nodes. One example of immunogenic DCs is “Tip DCs”. “Tip DCs” are large and granular DCs that are associated with bacterial, viral, and parasite infections and autoimmune diseases. They produce TNF- α and inducible nitric oxide synthase (iNOS) in response to TLR agonists.⁹ Regulatory or tolerogenic DCs are important in controlling inflammation, and preventing autoimmunity and fetal allograft rejection during pregnancy. The microenvironment also determines the function of regulatory DCs. IL-10, TGF- β , vitamin D, glucocorticoids, and other steroid hormones have been shown to generate regulatory DCs.¹² Regulatory DCs are able to regulate the immune response through a variety of mechanisms including IL-10 production or expanding regulatory T (Treg) cell populations.¹² Plasmacytoid DCs are a subset of DCs that produce large amounts of type I interferons (i.e. IFN- α and IFN- β) after stimulation by viral or microbial infections. Such plasmacytoid DCs are located throughout the body but are in high

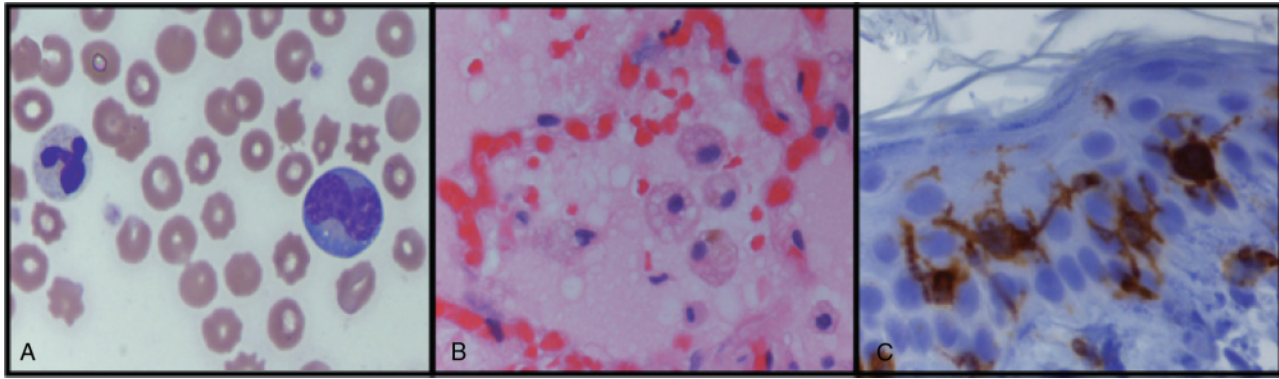


FIGURE 8.2 Characteristic morphology of monocytes, macrophages, and DCs in dogs. (A) Blood smear from a dog showing a neutrophil (left) and monocyte (right). Note the characteristic basophilic granular cytoplasm and irregularly shaped nucleus of the monocyte. Wright-Giemsa stain, original magnification $\times 1000$. (B) Pulmonary alveolar macrophages containing phagocytosed alveolar fluid and surfactant from the lung of a dog. Hematoxylin & Eosin stain; original magnification $400\times$. (C) Langerhans cells from skin of a dog. CD18 stain; original magnification $400\times$.

concentrations at immune sites exposed to the external environment (e.g. mucosal sites).

DIFFERENTIATING MONOCYTES, MACROPHAGES, AND DENDRITIC CELLS

DC can be round to stellate in appearance and can appear very similar to macrophages in tissue sections or aspirates. The prototypical “veiled” morphology with numerous thin projections resembling neuronal processes, used to originally define DCs, are difficult to appreciate in cytological and histological preparations. DCs in culture have limited or transient adherence and may resemble large lymphocytes with only small pseudopodia or dendritic processes. Upon activation or maturation, DCs demonstrate the more typical stellate morphology as seen in vivo in LCs (Fig. 8.2).

Monocytes, macrophages, and DCs express cell surface markers that are important in cellular function and useful in cell identification (see Chapters 4, 45, and 71). While many of these markers have been described in humans and mice, reagents in veterinary species are becoming increasingly available.^{3,14,15}

At present, there is no single marker that definitively differentiates monocytes, macrophages, and DCs. Rather, it is a combination of phenotypic markers, cellular morphology, tissue location, and functional characteristics that is used to identify cells of the MPS. CD14 expression is highest on monocytes and decreases as monocytes differentiate to macrophages or DCs. However, loss of CD14 expression on macrophages and DCs differs among species.^{3,15} Conversely, MHC class II, a molecule important in antigen presentation, is low in monocytes but is up-regulated with maturation and differentiation into macrophages and DCs. F4/80 is present on nearly all macrophage populations and is useful in fixed tissue sections.³ CD11b (Mac-1) is expressed on myeloid cells such as macrophages and myeloid DCs. CD11c is the most commonly used marker

to specifically identify DCs because monocytes and macrophages typically are negative for CD11c.⁶ However, CD11c is not a universal marker for all DCs because lung DCs and plasmacytoid DCs express little or no CD11c.⁶

SUMMARY

In summary, monocytes, macrophages and DCs are important hematopoietic cells that play critical roles in defense and maintaining homeostasis. Monocytes often are a direct indication of the health of the myelomonocytic lineage. Although the tissue locale of macrophages and DCs often limits extensive cytologic evaluation of these cells, function and dysfunction of these cells have important implications in health and disease.

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Thrombopoiesis

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Thrombopoiesis Overview

Megakaryocyte-erythroid Progenitor
Bone Marrow Endothelial Cells

Transcription Factors

GATA1 and GATA2
Friend of GATA1, Friend Leukemia Integration 1,
and GA-Binding Protein
Core Binding Factors
Nuclear Factor-Erythroid 2

Megakaryocyte Maturation and Platelet Formation
Endomitosis

Demarcation Membrane System and Proplatelet
Formation

Thrombopoietin and Other Key Hormones

Thrombopoietin

Stromal Cell-Derived Factor-1 (SDF-1)

Other Cytokines and Growth Factors

Megakaryocyte Ploidy and Platelet Mass

Acronyms and Abbreviations

Arp, actin-related protein; BFU-MK, burst forming unit-megakaryocyte; BMEC, bone marrow endothelial cell; CalDAG-GEFI, calcium diacylglycerol guanine nucleotide exchange factor I; CBF, core binding factor; CD, cluster of differentiation; CFU-MK, colony-forming unit-megakaryocyte; CXCR, chemokine receptor; DMS, demarcation membrane system; E#, day of embryonic development, where the number indicates age of the embryo in days after conception; FGF-4, fibroblast growth factor 4; FCR, receptor for the FC portion of immunoglobulin; Fli1, Friend leukemia integration 1; FOG1, Friend of GATA1; GABP, GA-binding protein; GATA1 and 2, GATA binding proteins 1 and 2; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; GP, glycoprotein; IL, interleukin; LIF, leukemia inhibitory factor; Lin⁻, lineage negative; JAK, Janus kinases; MAPK, mitogen activated protein kinases; Meg-CFC, megakaryocyte-colony forming cells; MEP, megakaryocyte-erythroid progenitor; Mpl, myeloproliferative leukemia; NF-E2, nuclear factor-erythroid 2; PECAM-1, platelet endothelial cell adhesion molecule-1; PF4, platelet factor 4; PIP4K α , phosphatidylinositol-5-P-4 kinase; SCF, stem cell factor; SDF, stromal cell-derived factor; STAT, signal transducers and activators of transcription; TPO, thrombopoietin; VCAM-1, vascular cell adhesion molecule-1; WASp, Wiskott-Aldrich syndrome protein; WAVE, WASp-family verprolin-homologous protein.

THROMBOPOIESIS OVERVIEW

Megakaryocyte-Erythroid Progenitor

Megakaryocytes are derived from a bipotent megakaryocyte-erythroid progenitor (MEP).⁶ Although the MEP was once thought to arise from a committed common myeloid progenitor, recent evidence suggests that the MEP may arise directly from an uncommitted short-term hematopoietic stem cell.¹ Primitive MEPs and megakaryocyte colony-forming cells (Meg-CFCs) have been detected in mouse embryo yolk sacs and give rise to primitive GPIIb β -positive platelets that are of large diameter, highly reticulated and relatively hypogranular.²⁶ These early platelets can be detected in circulation in mouse embryos as early as embryo day (E) 10.5. By day E11.5, definitive MEPs and Meg-CFCs within fetal liver become the predominant source of megakaryocyte pro-

genitor expansion and produce platelets that are more similar in size and granularity to adult platelets. It is not known whether primitive platelets produced within the yolk sac represent a population of platelets distinct from those produced by the fetal liver and ultimately the bone marrow because specific markers are unavailable.

Bipotent MEPs cannot be distinguished morphologically from small lymphocytes in bone marrow but have a unique cell surface protein expression pattern that includes interleukin (IL)-7R α ⁻/lineage negative (Lin⁻)/c-Kit⁺/Sca-1⁻ clusters of differentiation (CD)34⁺/FcR γ ^o.¹⁴ Once commitment to megakaryocyte lineage occurs, cells begin to express CD41 and CD61 (integrin α IIb β 3 or GP IIb and IIIa), CD 42 (glycoprotein Ib), and glycoprotein V. Two morphologically distinct colonies leading exclusively to megakaryocyte production have been identified in vitro: burst-forming unit-

megakaryocyte (BFU-MK) and colony-forming unit-megakaryocyte (CFU-MK).¹⁴

Bone Marrow Endothelial Cells

The bone marrow can be divided into two anatomical and functional entities with respect to development of hematopoietic cells: the endosteal zone, where non-dividing stem cells reside, and the more centrally located vascular niche that promotes stem cell differentiation and eventually mobilization of mature hematopoietic cells to the blood (see Chapter 3). Within the bone marrow, bone marrow endothelial cells (BMECs) support proliferation and differentiation of hematopoietic cells as a consequence of direct contact and release of cytokines. In turn, megakaryocytes nurture BMECs by secreting survival and growth factors. Platelet-endothelial cell adhesion molecule-1 (PECAM1) may play a role in movement of megakaryocytes toward the vascular sinuses. Transendothelial migration and proplatelet formation and release are dependent on the interaction of megakaryocytes with BMECs via adhesion molecules, including vascular cell adhesion molecule-1 (VCAM-1) and integrin $\alpha 4$ - $\beta 1$.

TRANSCRIPTION FACTORS

Several transcription factors are critical for normal thrombopoiesis in humans and mice that probably also regulate thrombopoiesis in other species.

GATA1 and GATA2

GATA binding protein (GATA1) is an X-chromosome encoded transcription factor that plays a key role in megakaryopoiesis and erythropoiesis. GATA2 is a closely related transcription factor that has early effects on maintenance of hematopoietic stem cells and multipotential progenitors.²⁸ GATA1 possesses distinct structural elements necessary for binding promoters and cofactors involved in progenitor cell development and differentiation. Several erythroid- and megakaryocyte-specific genes contain GATA motifs in critical *cis*-regulatory elements. For megakaryocytes these genes include those encoding for GPIIb, GPIb α , GPIb β , GPIX, platelet factor (PF)4, and the thrombopoietin receptor, myeloproliferative leukemia (c-Mpl). GATA1 activity is necessary for the divergence of megakaryocytic and erythroid progenitors from the MEP. Megakaryopoiesis is more sensitive to levels of GATA1 than erythropoiesis.²³ Several GATA1 mutations in humans are accompanied by a role reversal in which megakaryocytes are small and possess nuclei with reduced lobulation and erythroblasts are large and multinucleated.^{5,8}

Friend of GATA1, Friend Leukemia Integration 1, and GA-Binding Protein

Friend of GATA1 (FOG1) is an essential cofactor of GATA1. Mutations in GATA1 that result in impairment

of GATA1 binding with FOG1 result in X-linked macrothrombocytopenia in people. Friend leukemia integration 1 (FLI1) is a member of the Ets-family of transcription factors and interacts in a synergistic manner with GATA1 in the activation of megakaryocyte-specific genes. The heterodimeric GA-binding protein complex (GABP) is another Ets-binding factor that regulates gene expression in megakaryocytes. GABP, a complex composed of alpha and beta subunits, binds to promoters of early megakaryocyte-specific genes such as GPIIb and cMpl, whereas FLI1 primarily affects expression of late megakaryocyte-specific genes, including GPIb α , GPIX, and PF4. The alpha component of GABP mediates DNA binding of the complex whereas the beta component mediates transcriptional activation and nuclear localization of GABP α .²⁰ GABP and FLI1 both can bind to promoters of early and late megakaryocyte-specific genes and regulate activity of the GATA1/FOG complex. This overlap in function allows partial compensation if either activity is lost.

Core Binding Factors

A family of transcription factors referred to as core binding factors (CBFs) are critical in megakaryopoiesis and megakaryocytic differentiation. The CBF family contains three DNA binding proteins (RUNX1, RUNX2, RUNX3) and a non-DNA binding protein (CBF β). RUNX1 is a heterodimer composed of core-binding factor α -2 (CBF α -2) and CBF β . RUNX1 interacts with GATA1 and has an essential role in early hematopoiesis and vasculogenesis.²⁵ RUNX1 may also play a role in lineage determination. RUNX1 mutations in humans are associated with thrombocytopenia and platelet function abnormalities associated with decreased dense granule formation.¹⁷ In humans, CBF α -2 mutations have been associated with decreased levels of protein kinase C- θ ,²⁴ resulting in impaired activation of platelet integrin α IIB- β 3.

Nuclear Factor-Erythroid 2

Nuclear factor-erythroid 2 (NF-E2) is a key transcription factor involved in enhancing early megakaryopoiesis and in promoting megakaryocyte maturation. NF-E2 is a heterodimer consisting of a hematopoietic-specific p45 subunit and two p18 subunits, MafG and MafK. Mice with deleted genes for NF-E2 are severely thrombocytopenic, and megakaryocytes have reduced granules, a disorganized demarcation membrane system, fail to form proplatelets, and are unable to generate signals necessary for fibrinogen binding.²² Megakaryocyte targets for NF-E2 include genes encoding for thromboxane synthase, β 1-tubulin, Rab27b, caspase-12, calcium diacylglycerol guanine nucleotide exchange factor I (CalDAG-GEFI), and PINCH1. PINCH1 is an adaptor protein that interacts with integrin-linked kinase which in turn binds with cytoplasmic tails of integrins and thus may be important in mediating migration and interaction of megakaryocytes in the bone marrow microenvironment.³¹

MEGAKARYOCYTE MATURATION AND PLATELET FORMATION

In humans and domestic animals, megakaryocytes are located primarily in bone marrow. However, in adult mice, the spleen is considered a primary hematopoietic organ and generates all hematopoietic cell lineages, including megakaryocytes. Megakaryocytes undergo some degree of fragmentation in the pulmonary circulation in most mammalian species.

Endomitosis

During the early stages of megakaryocyte development, progenitor cells undergo typical cell division as they acquire lineage specific markers. In later stages of development, megakaryocytes begin several rounds of endomitosis in which anaphase, telophase, and cytokinesis are skipped, resulting in DNA division without cell division.²⁵ Mature megakaryocytes are polyploid cells and DNA content or ploidy correlates with the number of platelets a megakaryocyte will ultimately produce. The progression of megakaryocytes from dividing normally to dividing “atypically” is due to the lack of formation of a normal functioning mitotic spindle.¹⁰

Studies using a K562 erythroleukemia cell line suggest that down-regulation of stathmin expression may be necessary for induction of endomitosis in megakaryocytes. Stathmin is a phosphoprotein that promotes microtubule depolymerization and is phosphorylated and dephosphorylated in a cell cycle-dependent fashion. Stathmin is phosphorylated/inactivated as cells enter mitosis to allow for assembly of the mitotic spindle and then dephosphorylated/activated at the end of mitosis to allow dissolution of the mitotic spindle. Studies with K562 cells suggest that during early megakaryocyte development, stathmin expression levels are high, corresponding with normal cell division. During later stages of megakaryocyte development stathmin expression levels are reduced, resulting in abortive mitosis and polyploidization.¹⁰

Demarcation Membrane System and Proplatelet Formation

Terminal megakaryocyte differentiation occurs after endomitosis is completed and is associated with tubular invagination of the plasma membrane resulting in formation of the demarcation membrane system (DMS).²¹ The current model of platelet formation suggests that the DMS functions to provide a membrane reservoir for the formation and extension of proplatelets.²¹ Prior to proplatelet formation there is an accumulation of the phospholipid PI-4,5-P2 as a result of megakaryocyte lipid kinase activity. Specifically, phosphatidylinositol-5-P-4 kinase α (PIP4K α) is thought to generate PI-4,5-P2 by acting on the substrate PI-5-P. PI-4,5-P2 is thought to trigger actin polymerization by enhancing binding of Wiskott-Aldrich syndrome protein (WASp) to actin-related protein (Arp2/3). WASp-Arp2/3 complex for-

mation probably triggers Rho-family GTPases with activation of WASp-family verprolin-homologous protein (WAVE) proteins resulting in actin fiber assembly.¹⁹ Actin polymerization is associated with dilation and movement of the DMS to the cell periphery in preparation for microtubule assembly.¹⁹

Synthesis of platelet granules occurs simultaneously with formation of the DMS and granules are packaged and mobilized as microtubule assembly occurs in the extension of proplatelets. The microtubule motor kinesin is probably responsible for transport of organelles and granules along microtubules.¹⁸ Movement is bidirectional as microtubules slide past each other. Once organelles and granules enter the proplatelet tip they are captured there for packaging within the developing platelet.¹⁸ In addition to organelles, spliceosomes and pre-mRNAs are packaged into newly forming platelets, thus providing platelets with the capacity for protein synthesis.

Microtubule assembly is a dynamic process: time-lapse photography shows alternating extension and retraction as well as bending and branching of proplatelet shafts. Platelets form from the tips of proplatelet shafts; thus branching provides a mechanism for formation of more platelets per proplatelet extension. Branching and bifurcations of proplatelet extensions are mediated by actin forces. Elongation of proplatelets, however, is mediated by dynein, a molecular motor protein that mediates microtubule sliding necessary for proplatelet extension.

Microtubules are hollow polymers formed by α/β -tubulin dimers. β 1-Tubulin is the most abundant platelet β -tubulin isoform. β 1-Tubulin null mice attempt to compensate by up-regulating other β -tubulin isoforms, but other isoforms are unable to fully compensate. As a result, null mice have thrombocytopenia and platelets have reduced numbers of marginal microtubule bands. A missense mutation in β 1-tubulin causes macrothrombocytopenia in Cavalier King Charles Spaniels.

Proplatelets are formed in a spiraling manner starting at the periphery and moving centripetally towards the nucleus. Proplatelets consist of long beaded strands that fragment in circulation when subjected to shear. Shear stress within bone marrow sinusoids probably assists in fragmentation of proplatelets from megakaryocyte bodies after proplatelets are extended through the subendothelium. Activation of proplatelets is thought to be avoided by extension of large, cytoplasmic fragments into the lumen of sinusoidal vessels.¹¹ Proplatelet formation consumes the entire megakaryocyte cytoplasm leaving behind only the nucleus which enters an apoptotic pathway.

THROMBOPOIETIN AND OTHER KEY HORMONES

Thrombopoietin

Thrombopoietin (TPO) is a key humoral regulator of platelet production.^{3,15} TPO initially was identified as the

ligand for c-Mpl, the normal mammalian proto-oncogene version of a murine myeloproliferative leukemia virus.³⁰ The open reading frame for human TPO protein, including the signal peptide, encodes 353 amino acids. In cats, dogs, horses, cattle, pigs, and mice the open reading frame encodes 349, 352, 350, 349, 353, and 356 amino acids, respectively.^{3,16} At the amino acid level TPO is highly homologous across species and is 79%, 85%, 89%, 83%, 82%, and 81% homologous when comparing human to cat, dog, horse, cow, pig, and mouse, respectively.⁹ The amino-terminal end of TPO is highly homologous with erythropoietin in all species and is the portion of the protein that engages the TPO receptor. The carboxy-terminal domain is highly glycosylated which may be necessary for TPO survival in circulation and for secretion of TPO from cells. Truncated forms of TPO are found in circulation, particularly in thrombocytopenic animals.¹³ TPO is cleaved by thrombin at two sites, generating polypeptides that vary in activity. Cleavage of TPO at Arg¹⁹¹ in the carboxy-terminal domain results in a polypeptide with enhanced activity; however, activity is destroyed by thrombin cleavage at Arg¹¹⁷ within the amino-terminal domain.

Studies have documented that although TPO is critically important for normal megakaryopoiesis, TPO also is important for overall hematopoiesis and plays a role in enhancing expansion of hematopoietic stem cells.² TPO binding to c-Mpl results in activation of Janus kinases (JAKs) and signal transducers and activators of transcription (STAT) factors STAT3 and STAT5. JAK2 is bound constitutively to c-Mpl and upon activation phosphorylates tyrosine residues within c-Mpl and within STATs, PI3K, and mitogen activated protein kinases (MAPKs). STAT3 and STAT5 are required for regulation of normal megakaryopoiesis and have been found to be dysregulated in some myeloproliferative disorders and in megakaryoblastic leukemias in humans.

TPO is synthesized primarily by the liver and to a lesser extent in kidney and by bone marrow stromal cells. TPO receptors are located on platelets, megakaryocytes, and vascular endothelial cells. Although the numbers of TPO receptors on endothelial cells far exceed the numbers found on megakaryocytes and platelets, endothelial TPO receptors are not involved in regulation of TPO levels. Under steady-state conditions, TPO is produced constitutively and is bound by platelets and megakaryocytes via specific TPO receptors referred to as c-Mpl. Bound TPO is internalized and degraded and not available for stimulating thrombopoiesis above steady-state levels.

During episodes of thrombocytopenia, TPO levels increase, resulting in enhanced thrombopoiesis. The responsiveness of megakaryocytes to TPO is regulated somewhat by the maturity of the megakaryocyte. Megakaryocytes that are GPIIb+ GPIb+ undergo fewer endomitotic cycles in response to TPO compared to younger megakaryocytes that are GPIIb+ GPIb-. The enhanced endomitotic capability of GPIIb+ GPIb- megakaryocytes is due to lack of binding of 14-3-3 ζ adaptor

protein to the cytoplasmic tail of GPIb α . Lack of sequestration of 14-3-3 ζ results in enhanced phosphorylation and activation of Akt by phosphoinositol-3-kinase (PI3K). Activated Akt increases the post-translational stability of cyclins, which play a role in promoting endomitosis and increasing ploidy. Although TPO production is constitutive under steady-state conditions, severe thrombocytopenia can result in enhanced production of TPO by bone marrow stromal cells. Inflammatory conditions can result in enhanced TPO production by hepatocytes (mediated by IL-6) resulting in reactive thrombocytosis.¹² During certain pathologic conditions the steady-state mechanism of TPO production can be altered, resulting in TPO levels that are discordant with levels expected based on platelet numbers. In some cases of severe idiopathic thrombocytopenia, particularly where megakaryocytes are markedly increased in number and size, TPO levels may not be as high as expected due to binding of TPO to c-Mpl receptors on megakaryocytes.

Stromal Cell-derived Factor-1

cMpl-null mice are markedly thrombocytopenic but still capable of platelet production. Stromal cell-derived factor-1 (SDF-1) can function alone or synergistically with TPO to enhance thrombopoiesis and may be responsible for maintaining low level thrombopoiesis in *cMpl*-null mice. SDF-1 interaction with chemokine receptor 4 (CXCR4) may be responsible for movement of megakaryocyte progenitors to the vascular niche necessary for initiation of thrombopoiesis. Movement of megakaryocytes to the vascular sinusoid compartment of bone marrow, mediated by SDF-1 and fibroblast growth factor 4 (FGF-4), induces megakaryocyte maturation and platelet formation.⁵

Other Cytokines and Growth Factors

Although TPO is an important regulator of thrombopoiesis throughout all stages of megakaryocyte development, other cytokines act in synergy with TPO primarily in early stages of megakaryopoiesis. IL-3 synergizes with TPO to produce several hematopoietic lineages, but IL-3 effects are limited to the CFU-MK stage. Stem cell factor (SCF) and granulocyte-macrophage colony stimulating factor (GM-CSF) act in synergy to enhance proliferation and expansion of bone marrow progenitors and megakaryocyte lines. Granulocyte colony stimulating factor (G-CSF) combined with TPO can trigger hematopoietic stem cells to enter the cell cycle and support colony formation. In later stages of megakaryocyte development, G-CSF has been shown to suppress megakaryopoiesis. IL-11, IL-12, and erythropoietin also stimulate proliferation of megakaryocyte progenitors whereas IL-1 α and leukemia inhibitory factor (LIF) affect later stages of megakaryocyte maturation and platelet release. PF4 has been shown to inhibit megakaryocytopoiesis in vitro. PF4 is stored in platelet alpha granules; aberrant platelet activation within bone

marrow with subsequent PF4 release (e.g. radiation, chemotherapy) may contribute to thrombocytopenia.

MEGAKARYOCYTE PLOIDY AND PLATELET MASS

There are four to five times more megakaryocytes in mouse bone marrow than in human bone marrow, and mouse megakaryocytes are smaller. The difference in size is not related to ploidy distribution since the modal ploidy value for both species is 16N. Megakaryocyte modal ploidy is 32N for dogs and rabbits. In cats the modal value is evenly split between 32N and 64N.⁷ In 1 week old calves, the modal ploidy of cultured megakaryocytes was 64N, whereas in cattle aged 12–17 months the modal ploidy value was 32N.²⁷ Megakaryocyte ploidy is not consistently related to platelet mass and platelet mass is not constant across species. In dogs and cats, platelet size is inversely proportional to platelet number, whereas there is no correlation between platelet number and size in cows, goats, or ponies. Platelet number and size are directly proportional in horses. Mean platelet mass ranges from 1.8×10^6 to 2.5×10^6 fL/ μ L in cows, dogs, goats, and cats. In mice platelet mass is 5.2×10^6 fL/ μ L while in horses platelet mass is 0.72×10^6 fL/ μ L.⁴

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Lymphopoiesis

MARY JO BURKHARD

Immunologic Diversity

Combinatorial Diversity

VDJ rearrangement

Recombination signal sequences

Role of Rag 1 and Rag 2

Junctional Diversity

Terminal deoxynucleotidyl transferase

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Acronyms and Abbreviations

$\alpha\beta$, alphabeta; AIRE, autoimmune regulatory gene; BCR, B cell receptor; Blimp-1, B lymphocyte-induced maturation protein-1; CCR, chemokine receptor; CD, cluster of differentiation; DNA-PK, DNA-dependent protein kinase; Flt3, FMS-like tyrosine kinase 3; $\gamma\delta$, gammadelta; GATA3, GATA binding protein 3; HSC, hematopoietic stem cell; Ig, immunoglobulin; IFN γ , interferon gamma; IGF1, insulin-like growth factor 1; IL, interleukin; MHC, major histocompatibility antigens; NK, natural killer (cell); Rag, recombination activating gene; RSS, recombination signal sequences; TCR, T cell receptor; TdT, terminal deoxynucleotidyl transferase; TGF β , transforming growth factor beta.

Lymphopoiesis is the production and maturation of new lymphocytes. Much of what we know about lymphopoiesis has been derived from studies in genetically engineered mice. Although these pathways probably represent those seen in other species, there are known species differences in lymphocyte development, phenotype, and function.¹

Lymphocytes develop from a bone marrow derived pluripotent hematopoietic stem cell (HSC) that further differentiates into myeloid or lymphoid lineages. However, recent studies suggest that some lymphoid and myelomonocytic precursors maintain the capacity to differentiate along the other lineage pathway.⁵ The common lymphoid progenitor gives rise to three types of lymphocytes: B cells, T cells, and natural killer (NK) cells.

IMMUNOLOGIC DIVERSITY

Combinatorial diversity, junctional diversity, and somatic hypermutation are mechanisms that permit the immune system to generate B and T cells that detect a

large number of diverse antigens while maintaining a relatively small number of genes.

Combinatorial Diversity

Combinatorial diversity occurs in both immunoglobulin (Ig) and T cell receptor (TCR) genes through joining of different V, D, and J segments during VDJ rearrangement. Recognition of recombination signal sequences (RSS) by recombination activating gene (Rag) enzymes is crucial for VDJ rearrangement.

VDJ Rearrangement

VDJ rearrangement is a highly ordered, complex process requiring cleavage of DNA, addition or deletion of nucleotides to the cut ends of DNA, and finally DNA ligation. VDJ rearrangement is initiated by joining of one diversity (D) region with one junctional (J) region. A variable (V) region is then added and the intervening DNA is deleted, resulting in a single recombined V(D)J genomic sequence.

VDJ rearrangement defines specific stages of B and T cell development. In B cells, successful heavy chain rearrangement is required before light chain rearrangement can begin. Because kappa and lambda light chains lack a D segment, light chain rearrangement produces a VJ complex. In T cells, rearrangement of the TCR loci follows a specific order starting with delta, then proceeding through the gamma, beta, and finally alpha genes. Two distinct subpopulations of TCR expressing cells are formed from these chains: $\alpha\beta$ and $\gamma\delta$ T cells.

Recombination Signal Sequences

Ig and TCR gene segments are flanked by unique RSS containing a heptamer, a 12 or 23 base pair spacer, and then a nonamer. RSS orientation and spacer size are used during recombination to ensure orderly recombination and inhibit the recombination of similar gene segments (e.g. V-V).

Role of Rag 1 and Rag 2

Rag-1 and Rag-2 proteins are nuclear recombination enzymes that recognize RSSs and nick DNA to form hairpins where nucleotides can be added or deleted to create blunt-ended double-stranded DNA breaks. These ends are then aligned by the protein heterodimer Ku80/Ku86. Ku80/Ku86 are non-catalytic subunits of DNA-dependent protein kinase (DNA-PK). KU80/KU86 bring the broken DNA ends together for covalent ligation by the DNA ligase IV complex, which is critical for the DNA joining process in VDJ recombination. DNA-PK also recruits proteins for substrate phosphorylation, cleavage of the hairpin loops, and nucleotide addition. In general, Rag enzyme production ceases at the end of VDJ rearrangement. However, activated germinal center B cells can re-express the Rag genes during affinity maturation.

Junctional Diversity

Junctional diversity is created by the addition of nucleotides to the DNA ends that are cleaved during recombination.

Terminal Deoxynucleotidyl Transferase

Terminal deoxynucleotidyl transferase (TdT) is a DNA polymerase that adds variable numbers of nucleotides to the V, D, and J exons during rearrangement. These additional nucleotides at the VDJ junctions are called N regions and provide additional diversity of the Ig heavy chain and the TCR. TdT expression declines as B cells mature and thus does not increase light chain diversity.

Somatic Hypermutation

In B cells, diversity also is introduced through programmed hypermutation within the Ig variable genes when B cells are stimulated to proliferate. During hypermutation, uracils are introduced through cytosine

deamination and error prone DNA polymerases. This marked mutation within the B cell receptor locus enhances the development of diverse B cells that recognize novel antigens.

B CELL DEVELOPMENT

B cell lymphopoiesis is a life-long process that begins in the fetal liver and transitions to the bone marrow after birth. After leaving the bone marrow, B cells seed peripheral lymphoid tissues.

Growth Factors

In mice, the earliest B cells require soluble factors such as interleukin (IL)-7 as well as direct physical interaction with bone marrow stromal cells. It is likely that non-functional and autoreactive cells are deleted through these direct stromal interactions. IL-7 is not required for B cell development in people.⁴ Later stages of B cells require only soluble factors for development. IL-1 α , IL-3, IL-4, IL-11, transforming growth factor beta (TGF β) interferon gamma (IFN γ) insulin-like growth factor (IGF1), c-Kit ligand, FMS-like tyrosine kinase 3 (Flt3) ligand, and estrogen also are known to play a role in B cell proliferation, differentiation, and negative regulation.

Stages of B Cell Development

The earliest defined cells of B lineage are pro-B cells. These cells have limited proliferative ability and are characterized by rearrangement of the Ig heavy chain. As maturation proceeds to pre-B cells, a cytoplasmic heavy chain protein is produced that combines with a surrogate light chain to form a transient pre-B cell receptor (BCR). Expression of the pre-BCR inhibits further heavy chain rearrangement, stimulates cell replication, and is necessary for the survival and positive selection of pre-B cells. Light chain rearrangement occurs in late pre-B cells. Kappa rearrangement proceeds first. Lambda rearrangement will occur only if kappa rearrangement is unsuccessful. Light chain and heavy chain proteins combine to produce IgM. At this stage, cells also express cluster of differentiation (CD)10, CD19, and human lymphocyte antigen-DR (HLA-DR) markers.

As cytoplasmic IgM is exported to the surface membrane, cells transition into immature B-cells that express CD21. Surface IgM expression is used to delete cells reacting with self-antigen. Surviving immature B cells migrate to peripheral lymphoid tissues where they begin to express surface IgD as well as IgM. As these cells interact with antigen and T cells, they undergo the process of class switching, somatic hypermutation, and affinity maturation.

Class Switching (Isotype Switching)

Class switching is the process by which the mu and delta heavy chain constant regions are substituted with

constant region genes of IgG, IgA, or IgE. During this process, the heavy chain variable region and light chains remain the same; thus class switching results in the production of a different antibody class that has the same antigen binding area. Because IgG, IgA, and IgE have different Fc regions, these antibodies can be secreted or interact with different effector molecules. Heavy chain class switching is regulated by T cells through direct interaction and cytokine production.

T CELL DEVELOPMENT

T Cell Types

T cell development proceeds down two primary pathways as defined by expression of either an $\alpha\beta$ or $\gamma\delta$ TCR. In most species, approximately 90% of immature cells develop into $\alpha\beta$ T cells through thymic selection, while the remaining cells develop into $\gamma\delta$ T cells. $\alpha\beta$ T cells are CD3+ and the majority also will express either CD4 or CD8.

Natural killer T or NK1.1 T cells are a unique subpopulation of $\alpha\beta$ TCR expressing cells that express NK1.1, a marker typically found on NK cells. They are CD8 negative but may be either CD4 positive or negative. NK1.1 cells have a normal TCR rearrangement but then undergo selection via interactions with CD1 ligands in the cortical region.

T Cell Growth Factors

IL-7 is necessary for T cell development. Defects in the IL-7/IL-7receptor complex and signaling pathway are responsible for several reported T cell defects. Several transcription factors are important in T cell development. Notch1 is important for the transition from a common lymphocyte progenitor to a T/NK progenitor and, with GATA3, for the transition from the T/NK progenitor to sole T cell commitment.⁸ Notch1 alone directs cells towards $\gamma\delta$ TCR development, but HEB (one of the basic helix-loop-helix E-box binding proteins) directs cells towards $\alpha\beta$ TCR.⁷ B cell-induced maturation protein (Blimp-1), long established as critical for development of cells committed to the plasma cell lineage, recently has been shown to be essential for terminal differentiation of T cells.^{3,6}

Role of the Thymus

The thymus is seeded with lymphocytes during embryonic development. Thymic precursors enter at the cortico-medullary junction via molecules such as CD44, CD62P, and chemokine receptor 9 (CCR9), and begin to seed the subcapsular cortex. Marked T cell production continues until puberty at which time the thymus begins to involute. Developing thymocytes undergo both positive and negative selection (see Chapter 52). Greater than 95% of developing lymphocytes fail one of these selection processes. Thymocytes begin as CD4⁻CD8⁻ double negative cells, transition to double

positive CD4⁺CD8⁺ cells, and finally mature as single positive CD4⁺ or CD8⁺ T cells that are released from the thymus.

Thymic Stroma

The thymic stroma is a uniquely organized epithelial network on a mesenchymal cell backbone with interspersed dendritic cells and macrophages (see Chapter 52). This cellular network provides the extracellular matrix ligands and growth factors critical for T cell selection and development.

The Beta Selection Checkpoint

Expression of the TCR occurs during the double negative stage of T cell development. To survive the beta selection checkpoint, cells must express a functional beta chain, which results in combination of the TCR chains with CD3 to create a functional surface TCR complex. Rearrangement of the TCR beta chain is required for successful rearrangement of the TCR alpha chain and subsequent formation of the TCR. While TCR beta selection does not appear to be tightly coupled to CD4 or CD8 regulation, production of a functional TCR is needed for CD4 and CD8 expression and development of double positive cells.²

Positive Selection

Positive selection occurs in the cortex where double positive thymocytes interact with low to moderate affinity major histocompatibility antigen (MHC) peptide ligands on cortical epithelial cells to receive a survival signal. Cells that lack adequate affinity to bind the MHC ligand complexes undergo apoptosis. Although the basis of positive selection is the TCR-MHC interaction, CD4 and CD8 are also required for selection. Double positive cells that select MHCII molecules will become CD4 T cells while those that select MHCI molecules will become CD8 T cells. This forms the basis for lineage commitment.

During the initial interaction, TCR signaling induces down-regulation of both CD4 and CD8. CD4 is then re-expressed. CD4+ cells that recognize MHCII receive a second signal and persist as single positive CD4+ thymocytes. Cells that previously recognized MHCI will not receive a second signal which induces down-regulation of CD4 and up-regulation of CD8.

Negative Selection

Thymocytes that survive positive selection up-regulate cell-surface expression of the chemokine receptor CCR7 and migrate to the thymic medulla for elimination of autoreactive T cells through negative selection. Medullary epithelial cells are induced to express peripheral antigens (e.g. those expressed by the pancreas, liver, etc.) through the autoimmune regulator gene called AIRE. In the medulla, thymocytes interact with

high affinity self-ligands on the medullary epithelium. Cells that react strongly receive an apoptotic signal. Surviving cells undergo final maturation and then exit the thymus.

Extrathymic Development

Most $\gamma\delta$, and some $\alpha\beta$, T cells are generated at extrathymic sites after being seeded by HSCs during fetal development. Unlike $\alpha\beta$ T cells, $\gamma\delta$ T cells are not selected through gene rearrangement but rather have specific invariant gamma and delta combinations. Furthermore, many $\gamma\delta$ TCR expressing cells primarily interact with lipid antigens rather than peptide antigens.

NATURAL KILLER CELLS

NK cells are part of the innate immune system and are named because of their ability to kill cells lacking MHC I expression. NK development occurs in the fetal liver and thymus, and in the bone marrow after birth. While the earliest NK-restricted progenitor has not yet been clearly identified, T and NK cells share a common CD7+ precursor. Three stages of NK development currently are recognized: lineage commitment, NK receptor selec-

tion, and functional maturation. The transition from a common lymphocyte precursor to the T/NK precursor is mediated, at least in part, by Notch1 and the transcription factor Ets-1. Il-15 is required for NK growth and maturation.

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Vasculogenesis/Endothelial Progenitor Cells

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Terminology
Endothelial Progenitor Cells

Antigenic Determinants
Mobilization, Recruitment, and Homing
Clinical Relevance and Therapeutic Application

Acronyms and Abbreviations

Ang1, angiopoietin-1; CD, cluster of differentiation; CECs, circulating endothelial cells; CEPs, circulating endothelial progenitors; EPCs, endothelial progenitor cells; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; HGF, hepatocyte growth factor; HIF-1 α , hypoxia-inducible factor-1 α ; HSCs, hematopoietic stem cells; KDR, kinase insert domain receptor; MAPCs, multipotent adult progenitor cells; SDF-1 α , stromal derived growth factor-1 α ; VEGF, vascular endothelial growth factor.

The development of vasculature, is a complex multimechanistic process. This chapter introduces vasculogenesis and endothelial progenitor cells (EPCs). To lay the foundation for understanding this topic, this chapter begins with an explanation of terminology. The remainder of the chapter addresses phenotypical identification of bone marrow-derived EPCs and signaling events involved in mobilization and recruitment. Clinical relevance of these physiological processes in health and disease are briefly highlighted followed by an introduction to potential therapeutic applications and the promises that future investigation in this field may hold.

Terminology

The term *vasculature* encompasses both blood and lymphatic vessels. *Neovascularization*, and the less commonly used term *angiopoiesis*, refer to the development of new vessels. Neither term implies the mechanism by which new vessel growth occurs nor differentiate between blood and lymphatic vessel development. *Neovascularization*, however, has become accepted in the literature to indicate blood vessel formation whereas “lymphatic neovascularization” has been introduced to reflect development of lymphatic vessels.

Angiogenesis, by definition, is the sprouting of new vessels from a pre-existing vascular network.^{4,19} The term thus defines the mechanism of vessel production; however, is often used loosely. *Vasculogenesis* is the pro-

duction of new vessels via recruitment of bone marrow-derived endothelial progenitor cells.² As vasculogenesis is also the mechanism of vascular development embryologically, it is often further characterized as pre- or postnatal vasculogenesis. Like angiogenesis, vasculogenesis is currently accepted to reflect blood vessels. As research in the area of lymphatic vascular production progresses, new terminology will likely continue to develop (i.e. lymphatic vasculogenesis). *Lymphangioblasts* give rise to the lymphatic vasculature and *hemangioblasts* give rise to the blood vasculature. Currently, lymphangiogenesis is utilized as an all-encompassing term for lymphatic vessel development regardless of the mechanism.

Endothelial Progenitor Cells

Endothelial progenitor cells (EPCs), a recently recognized cell population in bone marrow and circulation, are bone marrow-derived cells that participate in vasculogenesis and vascular homeostasis. *Neovascularization*, occurs via two distinct mechanisms: (1) vasculogenesis (i.e. development of new vessels by recruitment of bone marrow-derived EPCs); and (2) angiogenesis (i.e. development of new vessels through sprouting of pre-existing vasculature). Although each individual process exists, neovascularization is likely combinatorial with both processes occurring simultaneously.²

ANTIGENIC DETERMINANTS

Endothelial progenitor cells were identified just over a decade ago.^{2,21} As such, phenotypical and functional characterization of this population continues to be elucidated. Hematopoietic cells and endothelial cells come from a common progenitor, the hemangioblast, present in both embryonic and adult life. Ontogenic development of these cells is dependent on expression of vascular endothelial growth factor receptor-2/kinase insert domain receptor/Flk-1 (VEGFR-2/KDR/Flk-1).^{6,20} In addition to VEGFR-2, cluster of differentiation (CD) 34 and AC133 (CD133/Prominin-1) also have been identified and are now accepted antigenic determinants for this cell population. EPCs, in both bone marrow and blood, have an accepted phenotype of AC133+/CD34+/VEGFR-2+.^{18,21} The stage at which hematopoietic stem cells (HSCs) and EPCs develop lineage commitment has not been clearly defined. However, it is recognized that EPC maturation results in rapid down regulation of AC133.¹⁸ It has been proposed that the AC133+/CD34+/VEGFR-2+ phenotype represents a more primitive EPC with high proliferative potential, which then gives rise to AC133-/CD34+/VEGFR-2+ EPCs with a more limited proliferative capacity.²⁴ AC133+ cells also have been found in a number of other tissues, as well as in acute and chronic myeloid leukemias and lymphoblastic leukemia.¹⁴ More recently, AC133 expression has been proposed as a marker for cancer stem cells, but this is controversial. The AC133 antigen is a glycosylation-dependent epitope of CD133. Thus, it is not synonymous with CD133 and its expression is restricted to undifferentiated cells, whereas CD133 has been shown to retain expression upon differentiation.^{10,23}

Other bone marrow-derived cells also contribute to vasculogenesis and vascular homeostasis and are considered subsets of EPCs. Myelo/monocytic CD14+ cells can give rise to an EPC-like cell, acquiring an endothelial cell phenotype, especially when influenced by factors such as VEGF and angiopoietin-1 (Ang1).^{7,22} Thus, CD14+ cells are considered to be a plastic reserve of EPCs with a phenotype that can be modulated based on their local microenvironment. Another subset of EPCs includes multipotent adult progenitor cells (MAPCs). Bone marrow-derived MAPCs phenotypically are AC133+/CD34-/VEGFR-2+/CD144(VE-cadherin)- cells which, when co-purified with mesenchymal stem cells can differentiate into multiple cell types, including endothelial cells.¹¹

In addition to circulating EPCs, mature terminally differentiated endothelial cells, which slough from the pre-existing vasculature, may also be present in the circulation. These are termed circulating endothelial cells (CECs). CECs increase in circulation subsequent to vascular injury yet differ from CEPs in that they have minimal proliferative capacity, contribute minimally, if any, to new vessel formation, and phenotypically are AC133-/CD146(P1H12)+.^{16,23} Similar to EPCs and CEPs, CECs can express CD34 and VEGFR-2.^{8,24}

MOBILIZATION, RECRUITMENT, AND HOMING

EPCs in homeostasis reside predominantly in the bone marrow. However, in response to specific signaling, EPCs are mobilized into circulation with recruitment and homing to designated tissues for vasculogenesis or vascular repair. Common conditions that induce mobilization of EPCs include ischemia, vascular trauma, and tumor growth.^{9,12,17} Initiation is dependent on secretion of pro-angiogenic factors such as VEGF, Ang1, stromal-derived growth factor-1 α (SDF-1 α), and granulocyte-macrophage colony stimulating factor (GM-CSF) from the tissue of interest.⁵ Tissue hypoxia, as occurs at sites of ischemic injury and in central necrotic regions of tumors, results in a marked increase in VEGF expression subsequent to up-regulation of VEGF transcription factor hypoxia-inducible factor-1 α (HIF-1 α).^{3,19} Once at the tissue site, EPCs may further perpetuate recruitment via their own release of VEGF, hepatocyte growth factor (HGF), granulocyte colony stimulating factor (G-CSF), and GM-CSF.

CLINICAL RELEVANCE AND THERAPEUTIC APPLICATION

EPCs have been isolated from circulation in health and disease, demonstrating their role in both physiological and pathological vascular development. In health, the number of CEPs is extremely low. Increased numbers have been identified in cardiovascular disease, vascular injury, ischemia, fracture repair, and neoplasia.^{1,9,15} As such, this cell population and their regulatory molecules can be exploited as biomarkers of disease, prognosis, or response to therapy.¹³

With a continued understanding of molecular mechanisms regulating mobilization, recruitment, and incorporation of EPCs into new vessels, modulatory therapies to either stimulate or inhibit vascular development carry the potential to have a significant therapeutic impact. Additionally, in conditions where increased vascular development is specifically desired (i.e. bone repair and regeneration, myocardial infarction, and ischemic injury), direct EPC therapy with or without exogenous stimulatory factors may also hold promise for the future.^{12,15}

This chapter is dedicated to Dr. Moses Judah Folkman, the Father of Angiogenesis, 1933–2008.

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SECTION II

Hematotoxicity
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Design and Methods Used for Preclinical Hematotoxicity Studies

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Regulatory Guidelines

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Flow Cytometry

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Acronyms and Abbreviations

CBC, complete blood count; CBER, Center for Biologics Evaluation and Research; CDER, Center for Drug Evaluation and Research; CFSAN, Center for Food Safety and Applied Nutrition; CO₂, carbon dioxide; CVM, Center for Veterinary Medicine; EDTA ethylenediaminetetraacetic acid; EMEA, European Medicines Agency; FDA, Food and Drug Administration; Hct, hematocrit; Hgb, hemoglobin concentration; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; MHLW, Ministry of Health, Labor and Welfare; O₂, oxygen; PLT, platelet; RBC, red blood cell; WBC, white blood cell.

The complete blood count (CBC) is the foundation of the evaluation of the hematopoietic system in preclinical toxicology studies and clinical trials in humans. The unique advantage of preclinical studies is that most studies are terminal, and therefore histopathological assessment of the bone marrow is routinely performed. Microscopic evaluation of the bone marrow, together with concurrent evaluation of the CBC, provides a more complete evaluation of the health of the hematopoietic system than assessing the CBC alone. Any xenobiotic-induced changes in the hematopoietic system can then be correlated to other microscopic changes in tissues. Ideally the same animals should be used for hematological and histological assessment so that these correlations can be made on an individual animal basis.³⁰ Because of the benefits associated with the concurrent evaluation of the CBC and the bone marrow it is not surprising that these two procedures are specifically mentioned in regulatory guidelines that outline the design of preclinical toxicology studies of pharmaceuticals. The goal of this chapter is to briefly review the regulatory guidelines pertaining to the evaluation of the hematopoietic system in preclinical toxicology studies, how and when to obtain samples, and the general approach to integration of the different hematological tests.

ology studies, how and when to obtain samples, and the general approach to integration of the different hematological tests.

REGULATORY GUIDELINES

Governmental agencies responsible for ensuring the safety and efficacy of health products, such as the United States Food and Drug Administration (FDA), European Medicines Agency (EMA) and Japan's Ministry of Health, Labor and Welfare (MHLW), have guidance documents for preclinical toxicology studies that contain recommendations for hematological testing, as well as bone marrow histological and cytological evaluation. The FDA has four centers that are involved in food and drug safety, including the Center for Food Safety and Applied Nutrition (CFSAN), the Center for Drug Evaluation and Research (CDER), the Center for Biologics Evaluation and Research (CBER), and the Center for Veterinary Medicine (CVM). The EMA and MHLW have several bureaus or committees that regulate medicinal products for human and veterinary use.

Overall these agencies have published similar recommendations for blood and bone marrow evaluation, although the amount of detail and guidance varies.

The types of toxicology studies that are needed for approval of new drugs consist of acute, subacute (or subchronic), chronic, and carcinogenicity toxicity studies. In acute toxicity studies, the test article is administered once at different doses followed by an observation period of 14 days.²⁴ Subacute and chronic toxicity studies are repeated dose studies where the test-article is administered multiple times at different doses for at least 2–4 weeks and for 6 or more months, respectively. Carcinogenicity studies are repeated dose studies where the test-article is administered for most of the lifetime of a rodent.

Hematologic evaluation is infrequently performed in acute toxicity studies. MHLW does not specifically request hematology assessment in single dose toxicity studies.³² In contrast, CDER guidelines indicate that if the acute toxicity study is to provide the primary safety data supporting single dose studies in people, clinical pathology and histopathology should be monitored at an early time, at maximal drug effect, and at termination.¹³ They do not define which parameters need to be assessed. In contrast to acute toxicity studies, hematologic testing is routinely performed in sub-chronic and chronic studies. The EMEA gives very broad guidelines for clinical pathology testing.^{8,12} Pharmaceuticals should be tested in two species, a rodent and a non-rodent, and both sexes should be evaluated in both species. For rodents and non-rodents, historical control data should be available for morphological, biochemical and physiological variables studied. The evaluation should be done at relevant time points to detect changes over time, and specific parameters to be measured are dependent on the animal species and study design. In addition EMEA refers to the recommendations of the Joint Scientific Committee for International Harmonization of Clinical Pathology Testing for which specific core hematological parameters to assess and the timing of these samples.³⁰ EMEA suggests that evaluation should be done in all animals in studies with low numbers of animals. In rodent studies specialized examinations may be performed in a subset of animals. Blood smears and bone marrow (sternebrae, femur or vertebrae) should be examined. Bone marrow cellularity should be assessed in at least one rodent study, although they do not define how this is to be done. Histopathology is done on all non-rodents, but in rodent studies is done first on control and high dose animals. If changes are identified in the high dose, additional dose groups should be examined to clarify the dose relationship. Bone marrow cytology is not mentioned. However, the International Conference on Harmonization (ICH) S8 guideline, which the EMEA adopted, states that bone marrow smear evaluation should be considered if unexplained alterations in blood or bone marrow histology occur.⁹

The MLHW guidelines for repeat dose testing are very similar to EMEA's guidelines.³² They recommend blood samples be taken before necropsy in rodents,

including interim sacrifice, and in non-rodents pretreatment samples should be evaluated. Although they recommend hematologic testing be done at the time of interim sacrifice of rodents, if these animals are in poor condition, many of the changes in the parameters may reflect secondary changes and thus may not be particularly useful in assessing the primary compound effect. In non-rodent studies of 1 month or longer duration, at least one interim blood sampling should be considered. Hematological parameters to assess, include red blood cells (RBCs), white blood cells (WBCs) including a WBC differential cell count, platelet (PLT) count, hemoglobin concentration (Hgb), hematocrit (Hct), and reticulocyte count. Histopathology recommendations include bone marrow (sternum, femur). There is no mention of bone marrow cytological examination. For veterinary drugs, the Japanese Food Safety and Consumer Affairs Bureau, indicates that blood should be collected for hematology in sub-acute and chronic studies from moribund and surviving animals.²⁴

FDA's Center for Veterinary Medicine has a recent draft guidance.¹⁶ It recommends hematology (RBC count, Hct, Hgb, MCV, MCH, MCHC, WBC count and WBC differential cell counts, and platelet counts) at several time points including the beginning and end of study. Two baseline samples are also recommended. Histopathology of bone marrow, site not defined, and bone marrow smears are listed as tissues to consider for microscopic examination. FDA's CFSAN has the most detailed recommendations for 3–12 month rodent studies and includes the same parameters listed above by the CVM, but the timing of the sampling is very well defined.¹⁵ Sampling for hematology should be done during the first two weeks of the study, midway through the study and at termination. Both bone marrow smears and reticulocyte counts (either automated or by microscopic evaluation) should be available on all animals and be used if there are effects on the hematopoietic system. Bone marrow histopathology should be done on the sternum. CDER and CBER do not have recent guidelines for hematologic testing in repeated-dose toxicity studies.

The guidelines governing the clinico-pathological evaluation of carcinogenicity studies vary between regulatory agencies. FDA and MLHW guidelines for drugs intended for humans, do not explicitly indicate that hematological assessment is mandatory.^{14,32} However, MLHW suggests that blood samples should be taken if appropriate to measure RBCs and WBCs, and to make blood smears in animals that are sacrificed before or at the end of the study. Blood smears may be useful to assess anemia or changes in lymph nodes, liver and spleen that may be present. For veterinary drugs, the Japanese Food Safety and Consumer Affairs Bureau indicates that blood should be collected for WBC and RBC counts from moribund and surviving animals. Blood smears should be examined in animals suspected of blood dyscrasia.²⁴ In contrast, the EMEA suggests that monitoring hematological parameters should be considered during the study and should be performed at study termination.¹⁰ This recommendation is in con-

trast to the one made by the Joint Scientific Committee for International Harmonization of Clinical Pathology Testing that stated that blood smears at unscheduled and scheduled terminations should be made to help in the detection and differentiation of hematopoietic neoplasia, but other hematologic testing is not recommended because of the variability of the data.^{29,30} Histopathological evaluation of bone marrow is expected in carcinogenicity studies.³²

There are two key hematological testing position papers, one that was endorsed by several international scientific organizations, for preclinical toxicology studies, both of which are very similar in their recommendations and are cited by regulatory agencies.^{12,29,30} The core parameters recommended include RBC count, Hgb and Hct, RBC indices (MCV, MCHC, MCH), RBC morphology, WBC count, absolute WBC differential counts and PLT count. In the absence of an automated reticulocyte count, blood smears should be prepared and examined, if needed, based on the test article effect on the hematopoietic system. At the time these guidelines were written, automated reticulocyte analysis was not easily accomplished, but now is a routine parameter. Bone marrow cytology smears should also be prepared from each animal at termination but only need to be reviewed if indicated on the basis of the test article effect on the hematopoietic system. These guidelines did not specifically address histopathological assessment of bone marrow.

PREANALYTICAL VARIABLES

General

Several preanalytical variables, including age, sex, source of the animals, fasting state, randomization and time of bleeding, anesthesia, diet, housing, animal, and sample handling should all be considered; controlling these variables will help ensure robust hematology data. First, animals of similar ages should be utilized. The rationale to maintain consistent age animals, especially in rats, is that hematological parameters change with animal age (e.g. RBC, Hgb, Hct, and neutrophil numbers increase, reticulocyte count decreases as animals mature).²³ Although dogs of similar age can be easily obtained, obtaining non-human primates of similar age can be more challenging, especially if wild-caught animals are used. Animals should also be obtained from the same commercial and geographical source because this can affect hematological parameters. For example *Cynomolgus* monkeys (*Macaca fascicularis*) from Mauritius, Philippines, or Indonesia have smaller RBCs than those from China or Vietnam and they are not affected similarly by *Plasmodium* sp. (W.J. Reagan, unpublished observation) which is endemic in this species.¹⁸

Randomization of the order of blood collection in toxicology studies also increases the uniformity of the data. This is especially true in rodents. It has been shown that transportation of mice within a facility to

mimic what may be done prior to collection of blood can increase plasma corticosterone and lead to decreases in lymphocyte counts.⁷ However, because the order of blood collection is sometimes determined by the dosing time, it is important to randomize the dosing order. If hematology sampling occurs on the same day as collection of a toxicokinetic sample, the hematology specimen should be taken first to minimize the effect of changes in hematology parameters due to repeated phlebotomy.

Inconsistencies in how the animals are handled can have a major effect on hematological parameters, especially for non-human primates where epinephrine-induced excitement responses readily occur. This results in a marked leukocytosis due to increases in lymphocytes and usually neutrophils. All these preanalytical variables will be minimized by a well-planned study that is carried out in a well-managed facility with appropriately trained personnel.

Fasted State

An important preanalytical variable to control is fasting. Fasting will be dependent on the objectives of the study, the species used, and the biological activity of the test compound.³⁰ Typically the in-life samples are collected from rats and large laboratory animals with an overnight fast (15–16 hours). Because rats are night feeders, an overnight fast is almost equivalent to 24 hours of fasting. Collecting samples in the fasted state will help increase the homogeneity of the data as well as minimize chances of potentially inaccurate analysis due to post-prandial lipemia. Thirty percent of non-fasted rat serum samples were found to be lipemic.²⁸ Fasting of rats will have significant effects on hematological parameters, mainly caused by hemoconcentration resulting in increases in RBC count, Hct, Hgb, and PLT count, but also decreases in WBC count and prolongation of prothrombin time and activated partial thromboplastin time.^{1,23} Although some publications have shown that clinical chemistry parameters are more homogeneous in fasted rats, who reach a non-absorptive state after a 16–24 hour fast, these principles are being challenged by animal care committees and thus some facilities are no longer fasting rats.²⁸ If the fasted state is chosen, animals should be fasted for each subsequent blood sampling period to allow for the best comparison. In contrast to large laboratory animals and rats, mice should not be fasted overnight as they tend to dehydrate rapidly; however, some facilities will use a 3–5 hour fast.¹⁷

Timing of Blood Collection

The timing of sample acquisition from the peripheral blood is somewhat dependent on the regulatory requirements, but also dependent on the goal of the study, the species of animal used, the blood volume of the test animal, the number of other samples needed to assess other parameters, the dosing time, and the results of previous studies. For repeated-dose studies in non-

rodent species, hematologic testing is recommended at study termination and at least once at an earlier interval. For studies of 2–6 weeks' duration in non-rodent species, testing is also recommended within 7 days of dosing initiation, unless it compromises the health of the animals.^{11,12,16,30} Additional sampling time points are included depending on the design of the study and should include the recovery period if present. In dog and nonhuman primates, one or two pre-study baseline samples are always taken. Measurements from the second baseline taken 2–3 weeks from the first one are often different as animals have acclimated to their new environment and there are less excitement-induced hematologic responses. Due to the small number of animals per dose group and the variability of the hematological results, the baseline sampling is critical to accurately assess any changes that occur during the study. The comparison of the in-life and end of study hematological results to the baseline sample(s) will allow for the most accurate assessment of the relevant changes in the hematological parameters. Hematologic testing is necessary at study termination in rodent repeated-dose studies. Interim study testing may not be necessary in long-duration studies if it has been done in short-duration studies using similar dose levels.^{11,12,16,30} Testing should be done at the end of the recovery period, if included.³⁰

In rodent studies, baseline sampling is not typically done. This is in part due to less variability in the hematological parameters of rats versus large animals. In addition, the number of rats used per dose group is greater (typically 10 rats per sex per group versus 3 or 4 per sex per group in large animal studies) which increases the confidence of the mean data in this species. The mean data of each dose group is then compared to the respective vehicle-control, although individual animal data should also be evaluated. In addition, in longer-term studies, the hematological parameters will change with age, which make comparison to pre-treatment values difficult.

Method of Blood Collection

In dogs, interim in vivo samples are taken from the jugular vein with manual restraint of the animals. Other sites including the cephalic and saphenous veins could be used. In non-human primates the animals are either manually restrained, typically with help of specialized chairs or cages, or anesthetized. The femoral vein is frequently used, but cephalic and saphenous veins can also be used. If chair restraint is used without anesthesia, it is critical to have a good acclimatization period for these animals before the start of the study to minimize the excitement of the animals during sampling.

Samples can be obtained terminally from large laboratory animals and are typically collected from the caudal vena cava, aorta or cardiac puncture. There are differences in hematological parameters depending on which vessel is sampled, so having the site of sampling consistent throughout the study is ideal.

Sites of interim in vivo sampling in rats are the jugular vein, lateral tail vein, tail artery, and sublingual vein, and these procedures can be done with either manual restraint or with anesthesia. Retro-orbital sinus bleeds have also been utilized, but due to animal welfare concerns this technique is not used frequently for in-life sampling. Anesthesia is recommended if the retro-orbital sinus technique is used.

In mice, limited amounts of blood can be obtained from the lateral tail vein or retro-orbital sinus. Anesthesia is recommended if the retro-orbital sinus technique is used. Some facilities are able to obtain a larger volume from the jugular vein with manual restraint or anesthesia. Coating the needle and syringe with a solution of 7.5% EDTA before sampling may help to avoid clotting. Terminal samples are taken at the time of necropsy from the caudal vena cava, aorta, or via cardiac puncture, and due to the greater volume these samples are easier to use for hematological analysis.

The use of anesthesia will have an effect on the hematologic results. Anesthesia is not typically used in dogs and may be used in non-human primates. If anesthesia is utilized in non-human primates, ketamine is typically used and will decrease WBC counts, mainly due to decreased lymphocytes.²⁰ Two of the more commonly used anesthetics for rats include isoflurane and different mixtures of CO₂ and O₂ (60/40, 80/20, 100/0 %).⁵ For example, when hematological results were compared between rats that had CO₂ anesthesia and no anesthesia, the most consistent results in both males and females exposed to CO₂ were increased MCV, increased Hct, decreased MCHC, and increase in WBC count, mainly due to lymphocytosis. With isoflurane anesthesia, male rats did not have significant effects on the hematology parameters compared to animals bled without anesthesia, but females had minor decreases in RBC count, Hgb, Hct, but no changes in WBC or PLT counts.

Amount of Blood Collected

The amount of blood that can be taken will be dictated by recommendations of the Animal Care and Use Committee at each facility. Although there are few studies to assess what are the ill effects of excessive bleeding, a good review on blood sampling has recently been published and makes recommendations on how much blood volume can be safely withdrawn.⁶ The blood volume is estimated to be 850 mL (85 mL/kg) in a 10 kg dog. Of this volume, 85 mL (10%) of the blood can be removed in a single sample or with multiple samplings over 24 hours if animals are allowed to recover for 2 weeks.⁶ Blood volume is more of an issue in monkeys due to the small size. A 5 kg *Cynomolgus* macaque would have 280 mL (65 mL/kg) of blood. Therefore, a total of 32 mL of blood (10%) can be removed in a single sample or with multiple samples over 24 hours if the animals are allowed to recover for 2 weeks.⁶ Although these volumes are adequate for routine hematological assessment, multiple blood samples taken for other analyses such as toxicokinetic

analysis, frequently result in reaching of maximal allowable amounts, and can lead to iatrogenic decreases in RBC mass.

Interim sampling can be done in rats, although the overall amount of blood and frequency of the bleeding will be limited. A 250 g rat has approximately 16 mL (64 mL/kg) of blood. Of this volume, a total of 1.6 mL (10 %) can be safely removed in a single sample or with multiple samples over a 24 hour period if animals are allowed to recover for 2 weeks.⁶ Typically samples for interim hematological analysis are collected in microtainer tubes (usually 0.5 mL) in order to achieve an adequate blood/anticoagulant ratio and enough sample to assess on most hematological analyzers.

Interim sampling in mice is infrequently done due to the limited blood volume. A 25 g mouse has 1.8 mL (72 mL/kg) of blood. A total of 0.2 mL (10.0%) can be removed in a single sample or with multiple samples over 24 hours if the animals are allowed to recover for 2 weeks.⁶

HEMATOLOGICAL ANALYSIS AND PARAMETERS

A few types of automated hematology analyzers work well for the laboratory animal samples used in preclinical toxicity studies. These analyzers should have multi-species software to ensure proper analysis because typically the automated WBC differential cell count features of these instruments are utilized. It is recommended that the blood be analyzed within a few hours after collection in all species. In addition, blood smears from all animals should be made soon after collection for possible review. Reticulocyte smears are not necessary if the hematology analyzer has been validated for reticulocyte counts. In dogs and non-human primates, making the blood smears within 2 hours after collection is sufficient for retention of good cellular morphology, although the sooner the slides are made after collection the better. In some strains of rats, if blood smears are not made immediately after collection there may be secondary artifacts of preparation such as crenation of the RBCs.

Each laboratory needs proper review criteria in place to ensure good quality hematological results from hematological analyzers. Although not clearly defined in regulatory guidelines, it is the primary author's (WJR) opinion that blood smears should be reviewed in all good laboratory practice-regulated studies. This review would include assessment of RBC, WBC and PLT morphology, as well as identification of other rare cell types. At a minimum, blood smears should be made from all animals in a study for potential review if warranted. Most laboratories that review blood smears, will often review all blood smears from the study. An alternative is to review blood smears from all large animals, due to the low number of animals per dose group, and have a tiered response for rat studies where high dose male and female rats as well as control samples are evaluated. If there are significant changes in morphol-

ogy then additional dose groups are evaluated. Another approach is to review a randomized subset of slides in high dose and control groups for all species.

The hematological parameters that should be measured are those recommended by the International Committee for Clinical Pathology Harmonization and include RBC count, Hgb and Hct, RBC indices (MCV, MCHC, MCH), RBC morphology, WBC count, absolute differential WBC count and PLT count.³⁰ Automated reticulocyte analysis should also be done as part of core hematology parameters on all studies. In addition RBC distribution width and mean platelet volume may sometimes be useful to assess changes in RBC populations and PLTs, respectively. The CBC should be evaluated in conjunction with the bone marrow histopathological analysis.

BONE MARROW HISTOPATHOLOGY

In large animals the sternbrae or rib are the preferred sites for histologic analysis because these sites will be hematopoietically active (see Chapter 132). In contrast, sternbrae and long bones of rodents, such as the femur, are frequently used. An overall assessment of bone marrow cellularity can be obtained with histopathology. The cellularity of bone marrow is dependent on the species, the site of collection, and the age of the animal. In addition, food restriction will affect bone marrow cellularity and blood cell counts.^{22,25} Concurrent vehicle controls and historical knowledge of what is typical cellularity of bone marrow for that aged animal and species is necessary to make an accurate assessment. If there are changes in cellularity, one should try to differentiate the cell types that are changed (i.e. myeloid versus erythroid). In large animals relative changes in the myeloid population compared to the erythroid population should be assessed if possible, but in rodents, due to the high percentage of lymphoid cells, differentiation of erythroid changes from lymphoid changes is difficult. Combining the evaluation of the blood results and histopathology results will allow for accurate assessment of the hematopoietic system.

BONE MARROW CYTOLOGY

Bone marrow cytological examination should be considered if there are significant or unexplained changes in the CBC and/or in the bone marrow histopathology results that need further clarification. Ideally, bone marrow smears should be made immediately or soon after euthanasia. Smears can be done by several different techniques, including push smears, pull smears and paintbrush technique (see Chapter 132). The slides made with any of the above techniques are air dried and then stained with a Romanowski-type stain such as Wrights or Wright-Giemsa stain.

Cytological analysis may consist of qualitative evaluation of the marrow smears alone and/or enumeration of the relative percentages of cells, typically by

doing a 300–500 cell differential cell count. Enumeration of hematopoietic cells can be done by detailed classification of the different cell types and stages of maturation or by classifying hematopoietic cells into the major categories of myeloid, erythroid, and lymphoid lineages. If the latter technique is used, a qualitative assessment can then be made of the maturation of the different lineages. Megakaryocytes should be noted as present or absent, but not enumerated. These cytological results will then be used in conjunction with the CBC and histopathological results to make an accurate assessment of the effect of xenobiotics on the hematopoietic system.

FLOW CYTOMETRY

An alternative to counting hematopoietic cells manually is flow cytometric evaluation of bone marrow. Flow cytometry provides rapid analysis of cell types present in the bone marrow.^{2–4,19,21,24,27,31,33} Percentages of the different cell lineages, and with some techniques a subdivision into proliferating and non-proliferating populations, can be assessed (see Chapter 132).³ These results are then interpreted in conjunction with the CBC and histopathology to determine which population is affected. In addition to these semi-quantitative assessments, a total nucleated cell count can be determined, and absolute counts of the different cell populations can be assessed (see Chapter 132). In contrast to only determining relative percentages of the different cell populations, absolute counts provide more definitive data to allow accurate assessment of which cell populations are truly affected.

OTHER TECHNIQUES

Rarely, other techniques such as bone marrow clonogenic assays or electron microscopy may be useful. Bone marrow clonogenic assays involve the *in vitro* differentiation of bone marrow using a mixture of growth factors to drive the differentiation of the different lineages. The advantage of this technique is to have an *in vitro* system to test the effect of xenobiotics on the specific bone marrow lineages and thus differential sensitivity of the bone marrow precursors can be assessed. This technique could then be used to assess a series of similar compounds to try to minimize toxicity. Electron microscopy, mainly transmission electron microscopy, is also rarely used when there is a morphological feature identified based on routine assessment of blood and bone marrow that needs further investigation.

In vitro hemolysis testing is commonly used to assess the potential direct hemolytic effect on RBCs of compounds that will be administered intravenously. Whole blood is typically incubated with various concentrations of test article and hemolysis is monitored either grossly or by measurement of RBC count and Hgb before and after incubation with the test article.

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Interpretation of Hematology Data in Preclinical Toxicological Studies

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Methods of Analysis – Good Laboratory Practice
 Regulations
 Reference Values
 Statistical Analysis
 Evaluation and Classification of Changes
 Hematology Changes
 Increases in Peripheral Blood Cells
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 Increases in leukocyte parameters
 Increases in platelet counts

Decreases in Peripheral Blood Cells
 Decreases in red blood cell parameters
 Decreases in leukocyte parameters
 Decreases in platelet count
 Adverse and Non-Adverse Effects
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 Correlation of Hematology Changes with Other Study
 Data
 Report Writing
 Conclusion

Acronyms and Abbreviations

EPO, erythropoietin; GLP, Good Laboratory Practice; Hgb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MPV, mean platelet volume; N, number of individuals per group; M, male; NOAEL, no observed adverse effect level; NOEL, no observed effect level; PLT, platelet; RBC, red blood cell; RETIC, reticulocyte count; RDW, range distribution width; SOP, Standard Operating Procedures; SD, standard deviation; WBC, white blood cell.

The principal objective of toxicological studies is to determine the potential toxicity of novel pharmaceutical compounds and chemicals. Many synthesized compounds are screened each year through the drug development process, but only one or two out of 10,000 reach the consumer market. Short term studies are initiated during the drug discovery process to help identify compounds with potential for development and quickly screen out undesirable molecules. These studies are designed with small numbers of animals and different doses of compound. Usually, only basic clinical pathology measurements are applied to these studies. Longer term studies are initiated to identify the target organs of toxicity, the lesions induced, the parameters that can be monitored to identify a developing toxicity, and the no observed adverse effect level (NOAEL) or no observed effect level (NOEL). These

studies typically involve one rodent species (mouse or rat), and one non-rodent species (e.g. dog, minipig, or non-human primate). They are tightly regulated by government regulatory health agencies (see Chapter 12).²⁰ Basic hematological, clinical chemistry and coagulation measurements are also applied to these studies, and bone marrow histopathological evaluation is performed in at least one of these studies.^{10,13,29,31} Further investigations, such as bone marrow cytology, are optional depending on the initial findings and evaluation of the hematology data.

Therefore, interpretation of hematological data is important at all stages of drug discovery and development. It is usually combined with the interpretation of clinical chemistry and urinalysis data, and with immunology and immunochemistry measurements if performed. Final interpretation and conclusion should

distinguish compound-related effects and biological variations. Results are part of a toxicology report and/or a clinical pathology report. These are written in collaboration with histopathologists and study toxicologists in order to provide not only a description of the study data, but also an accurate conclusion and an opinion on the significance of the changes.^{2,21,27}

METHODS OF ANALYSIS – GOOD LABORATORY PRACTICE REGULATIONS

Evaluation of hematology data should be done with knowledge of the inherent precision and variability of the assays used. Analytical data must be reliable and the laboratory should validate all analytical methods and operate internal and external control procedures. Agencies responsible for the investigation of the safety and efficacy of human health products regulate the conduct of non-clinical laboratory studies under good laboratory practice (GLP) regulations. GLP regulations provide the framework for performing scientifically valid studies and generating reliable safety data (see Chapter 12). GLP regulations must be applied to all non-clinical health and environmental safety studies required for registering or licensing pharmaceuticals, pesticides, food additives, cosmetic products, veterinary and drug products, and industrial chemicals. Some countries may have additional GLP regulations but differences in regulations are minor. There are memoranda of understanding with several countries whereby each country agrees to accept GLP studies and data from another country.⁶

The GLP clinical laboratory must have written approved standard operating procedures (SOPs), record systems, and quality assurance for sample handling, analysis, and reporting. A company employee or a consultant under contract may perform quality assurance activities. Quality assurance personnel audit the study protocol, and perform in-process audits of study conduct, including generation of clinical pathology data and evaluation of the report.

REFERENCE VALUES

Good Laboratory Practice and non-GLP laboratories currently establish reference ranges (or historical intervals) from healthy non-treated animals when new measurement methodologies are validated. Reference ranges can be calculated from non-treated control animals in toxicological studies. They are usually determined by the smallest and largest values if 20 or fewer animals are sampled or by the 2.5th and 97.5th percentile range if more than twenty animals are sampled (see Chapter 131). Consequently, reference ranges display values observed in 95% of a healthy population. Values outside the reference range do not necessarily indicate an abnormal condition and, conversely, values within the reference range do not imply a normal condition.¹⁹

Reference ranges are influenced by many variables, including species, strain, sex, age, animal supplier, animal husbandry or handling practices, diet and fasting, anesthesia, sample matrix, instrumentation, and test methodology. It is important to use reference ranges produced by the laboratory performing the study rather than published reference ranges. Animals used for establishment of reference ranges are most often housed, fed, and handled differently than those used in a particular study. Therefore, there is no substitute for age- and sex-matched control animals from the same supplier undergoing the same procedures as the test animals.¹¹ Reference ranges, however, are adequate to evaluate a single animal, pre-study data, data from controls, and to understand analyte variability. They may also be used to identify group means falling outside the reference range when no dose-response relationship or no correlating histopathological findings are identified.

Reference ranges should not be used as the only set of control values to determine potential test article effects on hematology data. In many cases, compound-related changes are small and post-treatment values fall within the reference range; therefore comparison of hematologic data to historical data, and to age-, sex-, and breed-matched control groups is essential.

STATISTICAL ANALYSIS

Laboratory data produced for a study may be voluminous, particularly for rodent studies. Statistical analysis of the data will frequently identify significant differences between control and treatment groups. These differences should be interpreted with caution, and not be used as a substitute for thorough review of the data. A change may be statistically significant but have little biologic or toxicologic significance.⁵ This is particularly true for hematology parameters that tend to reflect changes occurring in another organ or system.¹ Conversely, compound-related changes that are not statistically significant may be considered biologically significant. Both parametric and non-parametric statistical models are used in preclinical studies. Most parametric statistical models are suitable for data that are normally distributed and exhibit homogeneous variance. Non-parametric procedures are used when data are not normally distributed. The power of a statistical test is proportional to the number of animals per group. As the number of animals increases, more differences of small magnitude become statistically significant, although they may not be toxicologically relevant. Conversely, with small groups of animals, the numbers of statistically significant values tend to be low.

The mean or average is another less sophisticated tool that is appropriate for normally distributed data. Standard deviation describes the variability around the mean. When evaluating group hematologic measurements, evaluation of group means alone is not

sufficient, but standard deviations of each group should be carefully evaluated as an indication of variability.

EVALUATION AND CLASSIFICATION OF CHANGES

The main difference between the biopharmaceutical approach and veterinary medical practice approach in laboratory data interpretation is that toxicologic pathologists evaluate groups of animals and compare results with a vehicle-treated control group and/or pre-treatment values rather than evaluating individual animals. For most rodent toxicity studies, pre-treatment testing is not done. In non-rodents studies (e.g. monkey and dog), one or two sets of pre-treatment values are usually collected. When comparing data to pre-treatment values, comparison to the second set of pre-treatment data is usually preferred because these data are less likely to be influenced by stress, food intake, and other variables. In longer term studies, comparison with a concurrent control group is also important.³⁰ For example: if multiple blood samplings are performed during a study, RBC parameters of treated groups might vary if they are compared to their pre-treatment values, but differences in RBC values will not be observed if compared to control group values.

The objectives of these interpretations are:

1. To determine if there is a real difference between control and treated groups due to an effect of the compound or study procedure.
2. To differentiate the changes observed from analytical or biological variations.
3. To differentiate major and minor compound-related effects.
4. To determine if the compound-effect is related to the dosage.¹²

Biological variation is due to inter-animal components (i.e. differences between individuals) and intra-animal components (i.e. changes within an individual over time).⁵ Multiple physiological and procedural variables may influence hematology measurements, such as age, sex,²⁶ diet, fasting, restraint, circadian effects, blood sampling site,^{8,22,25} collection technique, order of collection, transport time, and sample preparation.¹² Sources of preanalytical variations should be avoided as these will broaden the confidence interval of results and interfere with interpretation. They must be considered and avoided when designing the study.

A treatment- or compound-related effect can be defined as follows:

A response to administration of a test compound in an animal that is considered to have substantial or noteworthy effect (positive or negative).

Although the principles of clinical hematology, as applied to veterinary patients with spontaneous disease, generally apply to the toxicological assessment of a drug, identification of very subtle effects which would not be noted on individual patients in veterinary care are frequently described as compound-related effects.

Several factors should be considered when determining a compound-related effect:

1. presence of a dose-response
2. presence of outliers which may influence the analysis
3. known class effects of the test compound
4. correlation with clinical chemistry data, clinical observations, necropsy and histopathological findings.

A *dose-response* is characterized by increased magnitude of the changes with increased treatment dosages. Identification of toxic effects in animals is generally expected to follow a dose-response pattern relative to incidence and severity, allowing the determination of dose levels where important or relevant effects occur, and dose levels where these effects do not occur. However, absence of a dose-response cannot exclude a compound-related effect. This is particularly true with immunostimulating drugs where compounds may have a dramatic effect in mid-dose groups, and little or no effect in the high-dose groups due to a saturation effect. In some cases overt toxicity and high mortality in the high dose group may mask a dose-related effect. Changes restricted to the high dose groups are considered to be dose-response effects. *Outliers* are extreme individual values (low or high) that are widely divergent from the mean of a group of data. These values may be due to a technical error, or directly related to another cause or disease considered non-compound-related. For example, an animal with renal disease might have moderate decreases of RBC parameters that would influence the group mean. In these situations, the group mean is affected and the standard deviation of the group is increased. Individual outlier values should be correlated to specific histopathological findings or clinical signs when possible. Differences identifiable only on the inclusion of outlying values are usually not considered to be dose-response effects, especially in studies involving large numbers of animals. In some instances, it may be helpful to recalculate the group mean with omission of the extreme values to determine whether an underlying trend remains. However, important individual changes should be reported if they are considered compound-related.

Transient effects are common. They should be reported and distinguished from transient procedure-related effects (e.g. increased reticulocyte counts following excessive blood sampling). Clinical pathology findings that are related biologically or that support a common pathophysiologic process should be grouped. Any evidence of an effect in control samples should also be reported.

Data interpretation has a subjective component. If a change is small, it is often interpreted as not important, and within pre-analytical or biological variability. As the change becomes larger, it is more likely to be significant and compound-related. However, there is frequently a range of results that are difficult to classify, and not possible to clearly differentiate as compound-related effects or biological effects. Therefore, these

changes may be reported as of uncertain relationship to the test article. Simplistic approaches to data interpretation such as statistics and comparison to reference ranges using a cut-off point decision method, with no consideration of a *gray zone*, may be a source of inappropriate decision making in the drug development process.⁴

HEMATOLOGY CHANGES

Minor effects of test-article effects on clinical pathology results are frequently observed. Clinical terms (e.g. anemia, leukocytosis) should be avoided unless major effects are observed and there is a deliberate attempt to underline an important effect. Compound-related effects are usually described as increases or decreases.

Increases in Peripheral Blood Cells

Increases in Red Blood Cell Parameters

Relative erythrocytosis is frequently observed with sub-clinical or clinical dehydration in preclinical studies, especially in animals with decreased food consumption or in rodents with prolonged fasting. With relative erythrocytosis, increases in RBC parameters (RBC count, Hgb, Hct) most often correlate with increases in total protein levels or albumin and/or urea. Absolute erythrocytosis resulting from a persistent increase in RBC mass and associated expansion of blood volume is rarely observed in toxicity studies, but may be seen when testing recombinant erythropoietin or other cytokines, or when the test article influences the production of erythropoietin.

Increases in Leukocyte Parameters

Increases in neutrophil and/or monocyte counts are observed with inflammation, excitement, steroid or stress response, or when testing pro-inflammatory cytokines.²⁴ Physiological leukocytosis in response to excitement can double leukocyte counts in minutes. In laboratory primates it is evenly distributed between neutrophils and lymphocytes. Increased neutrophil counts are most obvious in dogs, and increased lymphocyte counts are most evident in rats. Neutrophil counts increase in response to a variety of inflammatory conditions. Monocyte counts increase secondarily to any conditions with substantial tissue destruction, including inflammation, necrosis or hemolysis. Increases in lymphocyte counts may be observed with excitement in dogs and monkeys, and with chronic inflammatory lesions, especially in rodents, or with administration of antigenic compounds that elicit an immune response. Increases in eosinophil and/or basophil counts are infrequent in toxicological studies unless the test material is a hematopoietic growth factor or a cytokine. Leukemias are sometimes observed in 2-year carcinogenicity studies using rodents.^{12,28}

Increases in Platelet Counts

Physiologic increases in platelet (PLT) counts are usually transient, and result from increased mobilization of PLTs from storage pools during mild muscular activity or excitement. Healthy rodents have high PLT counts. Compound-related increases in PLT counts may be associated with blood loss, iron deficiency, or inflammation. Direct compound-related increases in PLT counts, presence of atypical PLTs in peripheral blood, and/or large numbers of megakaryocytes on tissue sections are rarely observed. Some test articles induce PLT dysfunction, characterized by normal PLT count, increased bleeding time and normal coagulation times.

Decreases in Peripheral Blood Cells

Decreases in Red Blood Cell Parameters

A frequent compound effect is a decrease in RBC parameters (RBC count, Hct, Hgb). The reticulocyte count is considered a reliable and early indicator of RBC regeneration when sufficient time has elapsed in species with circulating reticulocytes.³ Laboratory animals normally have low (dogs and non-human primates) to moderate (rodents) numbers of circulating reticulocytes. Decreases in RBC numbers with evidence of regeneration are either hemorrhagic or hemolytic, while decreases in RBC in the absence of regeneration indicate suppressed erythropoiesis. A frequent hematology finding in preclinical toxicology studies is mildly decreased RBC parameters with no evidence of regeneration. The animals may exhibit mild reductions in body mass and food consumption. Decreased food intake may have a major impact on the hematopoietic system and has been shown to decrease white blood cell (WBC), PLT, and reticulocyte counts.^{12,17,23} Numerous compounds impair erythropoiesis, either directly by injuring pluripotent hematopoietic stem cells, or their stromal microenvironment, or indirectly by inhibiting erythropoiesis (e.g. chronic inflammatory diseases).¹⁴

Decreases in Leukocyte Parameters

Leukocytes are frequently the first target of cytotoxic drugs because they have a shorter lifespan than PLTs and RBCs. In dogs and primates, marked acute inflammation and endotoxin-mediated pro-inflammatory cytokine release produces marked decreases in neutrophil counts, which is usually followed by a rebound neutrophilia. Recognition of decreased neutrophil counts is more difficult in mice and young rats that normally have low neutrophil counts. Immunotoxic drugs may trigger alterations in blood lymphocyte counts. Involution of the thymus is often interpreted as stress-related and correlates frequently with decreased lymphocyte counts. The significance of decreases in circulating monocytes, eosinophils, and basophils can be difficult to determine because these cells are normally present in low numbers and should not be over interpreted. Decreases in eosinophils or monocytes may be

observed with chemotherapeutic agents, and decreases in eosinophils are sometimes observed with stress or steroid-like compounds.

Decreases in Platelet Count

Decreased PLT counts are frequently associated with difficult venipuncture or PLT aggregation subsequent to inadequate blood coagulation, especially in dogs and rodents. Compound-related decreases in PLT counts can also result from impaired production or accelerated destruction or utilization. Test-material that reduces erythroid and myeloid production frequently inhibits PLT production as well. An example of hematology data interpretation is provided in Tables 13.1 and 13.2.

ADVERSE AND NON-ADVERSE EFFECTS

To be able to determine safe conditions for exposure in humans, it is necessary to identify in preclinical toxicological studies the lowest dose at which an adverse effect is first observed or the highest dose at which it is absent. For this reason, doses of exposure used in preclinical studies are based on multiples of the expected human dose. The clinical pathologist is expected:

1. To recognize and interpret hematology changes in the treated animals.
2. To determine if the effects are important toxic effects or minor responses to the drug.
3. To assist in the determination of the NOEL (i.e. the highest exposure level at which there are no effects observed).

TABLE 13.1 Group Mean RBC Parameters for a 13-Week Study With Daily Subcutaneous Injection of Human Recombinant Erythropoietin (EPO-R) in the Sprague Dawley Rat^a

Group	Summary Information	RBC ($\times 10^6/\mu\text{L}$)	Hgb (g/dL)	Hct (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	RDW (%)	PLT ($\times 10^3/\mu\text{L}$)	MPV (fL)	RETIC (%)	RETIC ($\times 10^9/\mu\text{L}$)
1	Mean	8.64	15.54	47.14	54.59	18.00	32.97	12.42	1126.2	7.54	2.68	230.16
	SD	0.42	0.84	2.56	1.03	0.47	0.6	0.54	107.1	0.27	0.34	32.18
	N	16	16	16	16	16	16	16	16	16	16	16
2	Mean	13.01	23.28	71.96	55.08	17.85	32.46	19.89	851.7	10.80	3.91	531.79
	SD	2.17	4.10	13.2	2.07	0.68	0.95	2.03	261.4	2.11	1.37	187.26
	N	10	10	10	10	10	10	10	10	10	10	10
3	Mean	15.04	22.43	75.44	50.32	14.98	29.78	23.01	1046.7	14.46	5.47	818.56F
	SD	1.379	1.88	5.62	2.88	1.04	0.83	0.99	361.4	4.93	0.26	68.98
	N	9	9	9	9	9	9	9	9	9	9	9
4	Mean	11.72	17.41	56.72	47.77	14.00	27.33	22.16	1400.97	16.09	6.48	964.92
	SD	6.16	8.53	33.00	2.44	0.95	2.94	4.19	728.32	8.71	4.84	714.28
	N	10	10	10	10	10	10	10	10	10	10	10

^aGroup 1 is the control group given vehicle control. Groups 2, 3 and 4 were given low, medium, and high doses EPO-R respectively. Interpretation of data: A treatment-related increase was observed in RBC, Hgb, Hct, and reticulocytes (RETIC) in groups 2 and 3 rats compared to control group. A treatment-related increase was also observed in RBC, Hgb, Hct, RETIC and PLT in high dose group (4) compared to control group. High standard deviations in group 4 were indicative of large individual variations within the high dose group (see Table 13.2).

TABLE 13.2 Individual RBC Measurements from High Dose Group Sprague-Dawley Rats Given Human Recombinant Erythropoietin^a

Group/Sex	Animal Number	RBC ($\times 10^6/\mu\text{L}$)	Hgb (g/dL)	Hct (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	RDW (%)	PLT ($\times 10^3/\mu\text{L}$)	MPV (fL)	RETIC (%)	RETIC ($\times 10^9/\mu\text{L}$)
4/m	4001	15.10	22.0	77.5	51.3	14.6	28.4	22.2	1784	26.3	8.2	1242.0
	4002	3.00	5.0	14.3	47.7	16.7	35.0	17.2	1153	8.1	0.7	19.6
	4003	2.61	4.2	12.7	48.5	15.7	32.3	18.6	1260	8.3	0.7	17.8
	4004	12.95	19.0	71.0	55.0	14.5	26.4	30.5	260	9.6	14.4	1866.0
	4005	15.20	22.0	18.0	51.4	14.5	28.3	23.4	1615	24.9	8.8	1328.2
	4006	15.30	20.5	75.0	49.1	13.5	27.4	22.7	2520	25.2	7.8	1205.0
	4007	16.10	23.0	81.0	50.3	14.3	28.4	24.4	2620	27.2	8.1	1308.4
	4008	2.6	4.1	12.5	48.1	15.6	32.5	17.9	1115	8.6	0.4	10.3
	4009	14.96	21.5	79.0	53.0	14.5	27.4	25.2	1470	20.0	9.4	1403.4
	4010	15.70	21.5	75.5	48.2	13.8	28.7	23.5	2700	23.8	6.6	1035.4

^aLarge individual variations were observed in RBC, Hgb, Hct, and RETIC. The majority of individuals had marked increases in RBC, Hgb, Hct and RETIC compared with control group, consistent with erythrocytosis and the pharmacological action of the compound. Some individuals (nos 4002, 4003, 4008) had a marked non-regenerative anemia due to anti-erythropoietin antibodies inducing an immune-mediated anemia in these individuals.

However, with a variety of agents, it is impossible to establish a no effect dose; therefore the term NOAEL is preferred. NOAEL is defined as the highest exposure level at which there is no adverse effect between the exposed population and its appropriate control. There is no standard definition for the term *adversity* from the literature and regulatory guidelines, although some and recommendations exist and have been published.^{9,16,18} One of the definitions proposed is:¹⁸

An adverse effect is a biochemical, morphological, or physiological change (in response to a stimulus) that, either singly or in combination, affects the performance of the whole organism or reduces the organism's ability to respond to an additional environmental challenge.

It may impair performance and generally have a detrimental effect on growth, development, or lifespan of a non-clinical toxicological model. More specifically, an adverse effect would be unacceptable if it occurred in a human clinical trial.

Alterations in hematology test results are infrequently the only evidence of important adverse toxicological effects. Clinical observations and pathology findings usually corroborate pathologically meaningful laboratory findings. Several factors can be used to differentiate an adverse effect from a non-adverse effect. An effect of treatment is less likely to be adverse if the severity of the change is minimal and not related to a change in other parameters. Non-adverse effects are usually adaptive or compensatory responses or findings that are below a threshold level. Effects that are adaptive responses to general chemical exposure will not be considered adverse. For example, increases in reticulocytes and bone marrow hyperplasia are induced by hemolytic drugs. Although changes in RBC count might be considered adverse, increases in reticulocytes and bone marrow hyperplasia will be considered an adaptive response to the compound-related decrease in RBC count. Reversibility is an important factor in the interpretation of toxicological studies. A change that is readily and completely reversible on cessation of treatment often indicates a lower level of concern, although it should not be used to discriminate adversity from non-adversity. Also, with potential new drugs, not every effect seen in non-clinical studies can be considered as unwanted or harmful, as some may result from the pharmacological action of the compound. For example, chemotherapeutic agents are usually cytotoxic and significant decreases in WBCs would be expected in non-clinical studies using healthy animals.

The NOAEL should be established for each study type for a particular compound. The initial determination of the NOAEL may change as additional information related to the molecule is generated. Data from long-term studies may give the toxicologist new or additional perspective as to whether or not an observation thought to be subtle or minor in a short-term study is an early indicator of a harmful effect. Species-to-species and within-species study-to-study variation may also occur. This may be due to normal biological variations or study design differences.⁹ Defining adversity depends on a precise interpretation of all data of a

preclinical study (e.g. food consumption, body mass gain, clinical signs, histology and immunology data, clinical biochemistry data). It is subject to discussion, challenge and reinterpretation.

TOXICOKINETICS

Toxicokinetics is a subdiscipline of pharmacokinetics exploring the absorption, distribution, metabolism, and elimination of xenobiotics at doses higher than those expected to produce therapeutic effects. Toxicokinetic evaluations are usually done in animal species used in safety assessment of drugs. They are determined directly from animals involved in non-clinical safety studies or from a designated toxicokinetic group of animals. The objectives of these measurements are:

1. To describe the systemic exposure obtained.
2. To determine the relationship between the dose and the toxicity observed.
3. To aid the determination of the margin of safety between non-clinical safety studies and human plasma concentrations achieved in clinical trials.
4. To judge inter-animal variability.⁷

Interpretation of hematological alterations must take into account the amount of drug-derived material to which the animals have been exposed and the route of exposure. Knowledge of the bioavailability of a drug and the kinetics of absorption, distribution and elimination are important to consider when interpreting hematology data.¹⁵ For example, it is sometimes possible to observe compound-related changes between groups that are caused by differences in drug exposure levels.

CORRELATION OF HEMATOLOGY CHANGES WITH OTHER STUDY DATA

The description of compound-related findings should include any association with clinical toxicology findings, any other clinical pathology data, gross pathology findings, body mass changes, organ mass differences and histopathologic findings. The correlation of compound-related hematology and histopathologic findings is particularly important as this plays a significant role in determining the NOAEL. Decreases in RBC parameters should be correlated with histopathologic evidence of hemorrhage, extramedullary hematopoiesis, and hepatic bile accumulation and/or hemosiderin deposits in liver and/or spleen. Lymphoid decreases often correlate with histologic atrophy of lymphoid organs. Increases in leukocytes may be correlated with microscopic evidence of inflammation in tissues. Decreases in blood cells should be correlated with bone marrow histopathology.

REPORT WRITING

Toxicology laboratories generally produce either an integrated final study report or separate pathology and

clinical pathology reports appended to the final study report. Each pharmaceutical company has its own way of presenting data, but as a general rule, data are presented in one or two tables including group and individual values for each treated group. Group values include mean, standard deviation, and statistical analysis applied to each group. Group and individual values are generally presented in a conventional order: control group given vehicle or group given the lowest dosage is presented first, followed by treated groups classified in increasing order of dosage (e.g. Table 13.1). When reporting the data, mean percentage differences or fold differences between treated and control groups can be used, and significance of the changes can be qualified by usage of modifiers: mild or slight, moderate or marked. When referring to fold difference, the method of comparison (e.g. pre-test values, second pre-test value, control group mean) should be stated.

CONCLUSION

Principles of hematologic interpretation used to characterize a disease in veterinary patients apply in preclinical toxicological studies. The main differences between these practices result from the evaluation of groups of animals rather than a single patient. Interpretation is based on an accurate utilization of reference values and statistical analysis, and correlation of hematology data with chemistry and urinalysis data, clinical observations, toxicokinetics, necropsy, and histopathological results. Clinical pathology data interpretation will help define the NOEL and/or NOAEL for each toxicology study in order to determine the potential toxicity of a drug for humans.

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Preclinical Evaluation of Compound-Related Cytopenias

LAURIE G. O'ROURKE

Compound-Related Cytopenias in Preclinical Studies

Predicting Hematotoxicity
Route of Exposure and Vehicles
Test Species

Mechanistic Classification of Drug-Induced Cytopenias

Drug-induced Immune (mediated) Cytopenias
Drug-induced Marrow Suppression
Anemia
Hypoproliferative anemia
Hemorrhagic anemia

Anemia secondary to induced suicidal erythrocyte death

Hemolytic anemia
Drug-induced non-immunologic adsorption of protein
Oxidative damage

Leucopenia
Thrombocytopenia

Disparaty between Preclinical and Clinical Safety Data
Investigative Work
Conclusions

Acronyms and Abbreviations

AIHA, autoimmune hemolytic anemia; AITP, autoimmune thrombocytopenia; BFU-E, burst forming unit erythroid; CFU-E, colony forming unit erythroid; CFU-GM, colony forming unit, granulocytic monocytic; DIA, drug-induced agranulocytosis; DIIMHA, drug-induced immune-mediated hemolytic anemia; DIMIT, drug-induced immune-mediated thrombocytopenia; GP, glycoprotein; IMHA, immune-mediated hemolytic anemia; IMT, immune-mediated thrombocytopenia; IgG, immunoglobulin G; IL, interleukin; PRCA, pure red cell aplasia; RBC, red blood cell; RDW, red cell distribution width; REDOX, reduction-oxidation reaction; TNF- α , tumor necrosis factor-alpha; TNFR, TNF- α receptor.

This chapter will focus on the susceptibility of the highly proliferative hematopoietic system to intoxication by exposure to xenobiotics or their metabolites. The ease with which blood can be sampled and analyzed makes it one of the most important target organs of the risk assessment process.

COMPOUND-RELATED CYTOPENIAS IN PRECLINICAL STUDIES

Predicting Hematotoxicity

Compound-related blood cell injury includes oxidative damage (see Chapter 36), non-oxidant mechanisms, immune-mediated damage (see Chapters 33, 38, 41, 78) and very rarely, induced apoptosis. Fortunately, in

silica high throughput systems are available that screen potential new chemical entities for known toxicities. This allows chemists to design and modify molecules to eliminate or mitigate the offending portions while retaining the active moiety. These designer compounds can then be screened for efficacy. With the exception of compounds deliberately designed to kill cells (e.g. anti-cancer drugs, antiviral drugs, pesticides, herbicides), prescreening of new chemical entities has greatly reduced or even eliminated the occurrence of hematopoietic toxicity. The intended purpose of the test molecule determines the acceptability of any impact on the hematopoietic system. For example, marketed antihypertensive drugs are extremely safe and effective when used properly but have been infrequently associated with adverse effects on the hematopoietic system. A potential new antihypertensive must be less toxic and

more efficacious than anything currently marketed to warrant development; therefore any evidence of toxicity targeting the hematopoietic system is unacceptable. However, hematopoietic toxicity produced by a molecule being developed as an anticancer drug, particularly when used as second line therapy after all other drugs have failed, might be considered acceptable.

The bioavailability of a compound provides detailed information regarding absorption, distribution, metabolism, and excretion, and in some cases, metabolite information, that can be helpful when evaluating compound-related effects. In particular, knowing the distribution, binding properties, metabolism, and $t_{1/2}$ of parent or active metabolites can aid in predicting both nadir and recovery of circulating cell counts before initiation of safety studies.

Compound-related effects can be directly or indirectly induced by the parent molecule (active molecular entity) or its metabolites. Secondary effects are consequences of direct or indirect toxic effect, such as renal failure due to nephrotoxicity. Less well understood or predictable are results that may be termed as hyperpharmacology. This form of adverse effect is frequently observed with recombinant molecules that have direct action on key biochemical pathways, thereby altering specific protein synthesis, secretion or catabolism, blocking key receptors, or gene regulation: this results in unpredictable consequences associated with induction of endogenous signaling molecules.

Compound-related effects can occur at one or more levels of hematopoiesis (from earliest stem cell to mature cells). Both *in vitro* and *in vivo* methods to define mechanisms of hematotoxicity or predict the degree of myelosuppression have been described. *In vitro* toxicity testing, using fresh primary target cells (hematopoietic and mesenchymal) from the species of interest, is now available through commercial sources.^{14,28} The author has found this approach invaluable for assessing comparative hematotoxicity between animal models and humans. *In vivo* mechanistic models, based on pharmacokinetic-pharmacodynamic data, are used to study the entire time course of myelosuppression and separate drug-specific parameters from system-related parameters, with the goal of optimizing dosing in clinical studies.¹⁵ The complexity of cytokine and hormonal effects on the bone marrow, peripheral cell counts, and cell function are the most unpredictable.^{3,36} Cytokines may induce changes in the myeloid population, the erythroid population, megakaryocytes, or other resident bone marrow cells, by up-regulation or down-regulation and may mediate these changes directly or indirectly. Changes are highly dynamic; therefore the timing of peripheral blood and bone marrow evaluations relative to dosing will produce different observations and interpretations. *In vitro* studies may be helpful in predicting cytokine release and establishing safety margins for clinical safety studies in healthy human cohorts. The use of transgenic mice has added significantly to our understanding of cytokine signaling pathways.³ For example, transgenic mice null for tumor necrosis factor- α (TNF- α) receptor

(TNFR) or double stranded RNA-dependent protein kinase receptor are protected from polycyclic aromatic hydrocarbon-induced bone marrow hypocellularity, demonstrating the role of TNF- α in this compound's mechanism of toxicity.²⁵

Route of Exposure and Vehicles

Each route of exposure will require safety testing and may introduce variables that can alter the effect of the compound on the hematopoietic system. Parenteral administration may prolong exposure. Intravascular administration introduces the molecule directly to circulating cells. Vehicles used for these routes may have no effect when tested separately, but may enhance or alter exposure to the test molecule. An example of this occurred in Europe with an upsurge in the incidence of antibody-mediated pure red cell aplasia (PRCA) in dialysis patients receiving recombinant human erythropoietin.¹³ This coincided with a change in the formulation, to comply with new regulations, where human serum albumin was replaced by glycine and polysorbate 80.²⁹ The variables that may have increased the immunogenicity of the product include formation of epoetin-containing micelles, interaction with leachates released from uncoated rubber stoppers of prefilled syringes, and denaturation or aggregate formation due to improper storage and handling. The incidence of PRCA returned to very low levels following replacement of the uncoated rubber stoppers with coated stoppers.

Test Species

Species that have been used most extensively are mouse, rat, dog, marmoset, and macaque. Baboon and chimpanzee have been used less frequently. Familiarity with the test species is important, as differences in response may occur between species (primates) and strains (rodents), and within species and strains based on vendor source. Some strains of rodents are genetically prone to hematopoietic abnormalities or have increased sensitivity to certain compounds, particularly knock-out animals. Extra care must be exercised when interpreting data from these animals and determining if an adverse effect is strain-specific.

MECHANISTIC CLASSIFICATION OF DRUG-INDUCED CYTOPENIAS

Drug-induced Immune (Mediated) Cytopenias

Preclinical observations are usually considered proprietary and are rarely reported in the public domain; thus, no estimate is available for the incidence of drug-induced immune cytopenias identified at toxicological exposures. Only 6–10% of all adverse drug reactions identified in clinical trials or post-marketing are recognized as idiosyncratic.¹⁸ Frequently, the mechanisms of these reactions are unknown, but the majority are believed to be immune-mediated. The incidence of

reported drug-induced immune-mediated hemolytic anemia (DIIMHA) in the human population is estimated to be one in one million individuals per year.⁵ Drug-induced agranulocytosis (DIA), defined as $\leq 0.5 \times 10^9/L$, is estimated at an annual incidence of 2–15 per million people and the mechanism may be direct toxicity, immune-mediated or a combination thereof.⁴ Drug-induced immune-mediated thrombocytopenia (DITP), in contrast, is more frequent.⁷

Idiosyncratic reactions appear to arise from a combination of genetic predisposition and environmental factors in susceptible individuals, and are frequently non-dose-dependent. A minimum of four events has been suggested as necessary for the initiation of an idiosyncratic reaction:

1. adequate amount of reactive metabolite(s)
2. binding with a protein with high epitope density
3. up-regulation of co-stimulatory signals on antigen presenting cells to activate T cells
4. failure of immune system to down-regulate a harmful immune response (see Chapters 16, 33, and 51).¹⁸

Sites of reactive metabolite formation usually correlate with the target of idiosyncratic reactions.¹⁸ For example, drugs oxidized by myeloperoxidase to reactive metabolites may induce immune-mediated agranulocytosis.¹⁸ It has also been suggested that glucuronide conjugation of metabolites may increase immunogenicity.¹¹

There are two types of drug-induced antibodies: drug-independent antibodies and drug-dependent antibodies.⁵ No drug is required in the test system for the detection of drug-independent antibodies, which may be “true” autoantibodies or antibodies that react like autoantibodies *in vitro* and can be found in combination with drug-dependent antibodies. The mechanism of drug-independent antibody production is unknown, but is hypothesized to be the result of a direct effect by the drug or its metabolites on the immune system that induces an autoimmune disease. Reactions attributed to “true” autoantibodies are detectable by direct antiglobulin test (Coombs’ test). Well documented cases of autoimmune hemolytic anemia (AIHA) have been induced by α -methyl dopa and procaine amide.⁶ Heavy metals are also incriminated, and depot injections of gold salts have often induced chronic autoimmune thrombocytopenia (AITP).³⁵ The drug-dependent antibodies require the presence of the drug in the test system for their detection and are frequently described as “hapten type”, “hapten-induced”, “penicillin-type” or “drug adsorption type”.⁶ Hemolytic anemias induced by this type of antibody response are associated with a high immunoglobulin G (IgG) titer and extravascular RBC destruction. This mechanism may also be responsible for some types of drug-induced thrombocytopenia.⁷ A second type of drug-dependent antibody, associated with complement activation and acute, severe intravascular hemolysis, is referred to as “immune complex type” or “non-penicillin type”. A proposed mechanism is that drug and/or metabolites

interact with the cell membrane (without covalent bonding) and lead to a polyclonal response to several epitopes. Antibodies that react with epitopes shared with the drug and the cell membrane produce immune complexes that trigger the reaction.⁵ The fibrinogen receptor, glycoprotein IIb/IIIa, von Willebrand factor receptor, or GPIIb/IX, may become selected epitopes, but the mechanism is not hapten-specific.⁶ Immunoglobulins only react with these platelet membrane glycoproteins when drug or sensitizing metabolite is present in soluble form. How the drug facilitates antibody binding to cause platelet destruction or how the immune system is triggered by the drug to recognize these epitopes is not understood.

Drugs developed as ligand-mimetic GPIIb/IIIa inhibitors have produced acute and often severe thrombocytopenia in a subset of patients during clinical trials.¹⁰ Naturally-occurring antibodies present in otherwise normal patients recognize GPIIb/IIIa complexed with the drug on the surface of platelets and produce DITP.

Drug-specific antibodies may be naturally occurring or induced. The cephalosporins are particularly prone to producing this response, and the problem has become more frequently observed with the newer generations of the drug. These DIIMHA can be very severe, with hemoglobinemia and hemoglobinuria. Differences in response to these drugs are found between age groups, with children often developing severe hemolysis within minutes after administration while similar problems occur in adults only after several days.⁵

Drug-induced immune disease against granulocytes has been associated with:

1. no significant effect on circulating numbers of neutrophils
2. moderate to severe neutropenia with normocellular to hypercellular bone marrow, or
3. neutropenia with bone marrow hypoplasia or maturational arrest.¹²

Identification of anti-neutrophil antibodies using various detection methods is difficult because they tend to be present in low titers and bind with low avidity. Autoantibodies that have been characterized in humans are directed against various membrane glycoproteins, including adhesion molecules. Anti-neutrophil antibodies can produce neutrophil dysfunction resulting in defects in adhesion, aggregation, chemotaxis, phagocytosis or killing.

Aplastic anemia may be a predictable or idiosyncratic reaction that presents as pancytopenia (leukopenia and thrombocytopenia will be detected before anemia) due to bone marrow failure (see Chapter 39). Radiation toxicity is the classic example of a dose-related, predictable aplastic anemia hematotoxin, but most drug-related cases are believed to be immune-mediated.⁹

PRCA is an infrequent idiosyncratic drug reaction in which marrow suppression is limited to the erythroid lineage. Although selective suppression of erythropoiesis may be the cause, an immune-mediated

mechanism has been speculated as the mechanism behind PRCA.

Drug-induced Marrow Suppression (see Chapters 17 and 18)

In addition to direct cellular injury, hematopoiesis may be suppressed or enhanced by alterations in the cytokine network. For example, based on the *in vitro* observation that interleukin-12 (IL-12) works synergistically with the c-kit ligand to promote hematopoietic stem cell proliferation, mice dosed with recombinant mouse IL-12 were expected to develop accelerated myelopoiesis.³³ Instead, treatment with IL-12 produced myelosuppression in bone marrow while enhancing splenic extramedullary hematopoiesis. The complexity of the cross-talk that occurs in the hematopoietic microenvironment is not restricted to compounds designed to impact hematopoiesis or regulate inflammation. Cytokines, growth factors, and receptors in tissues outside the hematopoietic system that are seen as potential drug targets may impact bone marrow as well. Blockade of neurokinin receptors has been pursued as a potential anti-anxiety target by allowing accumulation of Substance P. However, the same receptors exist in the bone marrow and play a critical regulatory role in hematopoiesis.²⁷ Depending on the receptor and the cross-talk, Substance P can either inhibit or stimulate hematopoiesis.

Anemia

Anemia may be caused by decreased circulating lifespan, hemolysis, hemorrhage, blood loss, decreased or ineffective production, or a combination of processes. The mechanism(s) of toxic effect(s) determines the type of anemia, time to onset, duration of effect, and whether persistence of the inciting agent is required for anemia to be maintained or progress.

Hypoproliferative Anemia

Mild normocytic normochromic anemia that is poorly regenerative and reverses when administration of the compound is discontinued is frequently seen in repeat-dose toxicity studies. Usually, histological and cytological evaluation of the bone marrow from these animals is unremarkable and there is no impairment of leukocyte or platelet production. Decreased erythropoiesis may be secondary to inanition associated with inappetence and other systemic toxicological effects of the administered compound.²¹

Compounds that have toxic effects on proliferating bone marrow cells or stromal cells may produce a non-regenerative or hypoproliferative anemia. Examples include anti-neoplastic drugs that target DNA and drugs that interfere with hemoglobin synthesis (see Chapters 16–18).

Hypoproliferative anemia may also occur secondary to decreased availability of iron, and may be induced by chronic administration of xenobiotics (e.g. recom-

binant cytokines or cytokine receptor inhibitors) that induce chronic inflammatory changes (see Chapter 37).³⁰ Long term studies with compounds that produce renal toxicity, such as cadmium, will also present with anemia due to decreased erythropoietin.¹⁷

Hemorrhagic Anemia

Hemorrhagic anemia, caused by blood loss, may be a direct or indirect effect of a xenobiotic. Acute hemorrhagic anemia, occurring over 2–3 days, will present with a decrease in the erythron and no evidence of regeneration. Thereafter, a regenerative response (polychromasia, anisocytosis, increased RDW, reticulocytosis) is generally observed. More protracted hemorrhage may present as regenerative or non-regenerative, or with characteristics consistent with iron deficiency anemia (hypochromasia, microcytosis, poikilocytosis). Changes in platelet counts and plasma protein concentrations will vary depending upon cause, duration, severity, and whether blood loss is internal or external. Mechanisms of compound-related hemorrhage include enhanced fibrinolysis, impaired platelet function, single or multiple coagulation factor deficiencies, and/or coagulation factor inhibition.²³ Secondary or indirect compound-related hemorrhage may occur as a result of compound-related gastrointestinal lesions, impaired vitamin K absorption, or disseminated intravascular coagulopathy. Examples of compound classes that may be associated with hemorrhage include non-steroidal anti-inflammatory drugs (secondary to gastrointestinal erosion, ulceration secondary to local irritation, inhibition of prostaglandin synthesis), vitamin K antagonists, platelet aggregation inhibitors, and gene therapy molecules (L.G. O'Rourke, personal observations).

Anemia Secondary to Induced Suicidal Erythrocyte Death

The normal wear and tear on RBCs exposes them to osmotic shock, oxidative stress and energy depletion. Eventually the cell is no longer able to perform adequately and is removed from circulation.²⁰ RBCs have their own process of programmed cell death which has been termed eryptosis because it mimics apoptosis.¹⁹ In addition to the triggers mentioned above, *in vitro* eryptosis can be induced by a number of endogenous molecules (e.g. ceramide, prostaglandin E2, platelet activating factor) and many xenobiotics (paclitaxel, azathioprine, amantadine, amiodarone, chlorpromazine, cyclosporine, methyl dopa, retinoic acid, cisplatin and zidovudine).¹⁹ The degree to which this mechanism may contribute to drug-related anemia has yet to be determined, but the hypothesis of drug-induced eryptosis is very compelling, particularly in circumstances where RBC lifespan appears to be reduced without explanation.

Hemolytic Anemia

Direct effects on circulating RBCs can produce acute or chronic hemolytic anemia. Hemolysis may be intravas-

cular, extravascular, or both, depending upon the insult. Intravascular hemolysis is accompanied by hemoglobinemia and hemoglobinuria. When damage is due to direct contact of a compound or its metabolite with the RBC, hemolysis is immediate and often severe. Examples of compounds capable of producing direct hemolysis include saponin, phenylhydrazine, arsine, or naphthalene.³¹

Drug-induced Non-immunologic Adsorption of Protein

Non-immunologic protein adsorption onto the RBC membrane, possibly influenced by the patient's plasma IgG concentration, is a possible mechanism of hemolytic anemia.^{6,27} Some drugs may be able to modify the RBC membrane, allowing adsorption of plasma proteins (e.g. immunoglobulins, albumin, complement, fibrinogen).³² Unfortunately, diagnostic tests are not available to differentiate this mechanism from immune-mediated mechanisms.⁵

Oxidative Damage Strong oxidants are able to overwhelm the capacity of RBCs to remain in a reduced state, resulting in the production of methemoglobin and/or denatured hemoglobin (see Chapters 16 and 36). Oxidation results in Heinz body formation and altered ion transport with subsequent intravascular hemolysis and removal by the mononuclear phagocyte system.^{9,31} Many species will form Heinz bodies when exposed to aniline, nitrobenzene, and related homologs. Blood film should be examined for evidence of Heinz bodies, eccentrocytes, and keratocytes. Unless there is continual exposure, it may be difficult to detect Heinz bodies in some species due to their rapid removal by the mononuclear phagocyte system. The ability of a molecule to function as an oxidant depends upon the concentration of exposure, the reducing ability of the RBCs of the species involved, and the susceptibility of the hemoglobin molecule. Obtaining an accurate measurement of methemoglobin is difficult due to the constant REDOX activity in the RBC, particularly in rodents. Some species are more susceptible to methemoglobin formation than to Heinz body formation, other species demonstrate the reverse, and still others are highly susceptible to both. Human RBCs have a relatively slow rate of methemoglobin reductase relative to laboratory species, and are particularly susceptible to Heinz body formation;³¹ therefore detection of either or both in a preclinical study is a major toxicological finding that must be carefully weighed against potential benefits of the compound.

Leukopenia

Drug-induced leukopenias may be transient or persistent. Transient depressions in leukocyte counts that return to baseline or are comparable to concurrent control data despite continued exposure to the compound may represent various compensatory mechanisms. Leukocyte depressions that persist throughout the dosing period of the study but return to baseline at the end of the study represent reversible suppressions.

Drug-induced neutropenia may be due to direct damage to progenitor cells in bone marrow, or damage to stromal cells that support granulopoiesis.¹⁶ Marrow damage may be limited to granulopoiesis, or affect all cell lineages. Mechanisms by which cytokines modulate the hematopoietic system are being continually studied because in vitro effects cannot predict the complex synergistic or antagonistic interactions within the cytokine network in vivo.³⁴ Time of evaluation can greatly influence interpretation. Some drugs, such as clozapine, may interact with intracellular myeloperoxidase or NADPH-oxidase to produce highly reactive products that are toxic to granulocytes.²⁴ Neutropenia can occur after prolonged exposure, and may be due to decreased production secondary to hypoplastic bone marrow.²² Immune-mediated granulocytopenia may be directed against precursor cells and/or against circulating cells. Criteria established to define idiosyncratic drug-induced agranulocytosis include:

1. neutropenia ($\leq 0.5 \times 10^9/L$)
2. onset during treatment or within 7 days of exposure with recovery within 1 month of discontinuation
3. recurrence upon re-exposure.^{4,8}

A recent review of non-chemotherapy drug-induced agranulocytosis found the median duration of exposure before onset ranged from 19 to 60 days, though one drug (dipyrene) had a median exposure of only 2 days.² Agranulocytosis caused by nonimmunological mechanisms appeared to have a later onset than when attributed to immune-mediated mechanism. Impaired granulopoiesis was responsible for 67% of the cases reviewed. This finding contrasts significantly with drug-induced thrombocytopenia or drug-induced hemolytic anemia, where blood cells are the main target of the immune reaction.^{5,7}

Thrombocytopenia

Thrombocytopenia may be a result of generalized bone marrow suppression, increased consumption, sequestration, or immune-mediated platelet destruction. Thrombocytopenia in preclinical studies is rarely associated with gross or microscopic hemorrhage unless there is concurrent platelet dysfunction. Although the most frequent cause of thrombocytopenia reported in clinical patients is drug-induced, thrombocytopenia is not a common finding in preclinical studies (L.G. O'Rourke, personal observation).^{5,9,31} Compounds that indiscriminately attack actively dividing cells will produce bone marrow panhypoplasia. Selective megakaryocytic toxicity, either through toxicity or an immune-mediated mechanism, is associated with a paucity of megakaryocytes in bone marrow. Conversely, with platelet destruction or consumption, as happens with DIMT, the marrow will show a marked increase in megakaryocyte numbers. Because the majority of reported drug-related thrombocytopenias are idiosyncratic and occur post-marketing, preclinical predictability of this important adverse effect remains underestimated. Any signal in the data from preclinical

studies that suggest that platelets are a target should be carefully and fully evaluated.

DISPARITY BETWEEN PRECLINICAL AND CLINICAL SAFETY DATA

Preclinical studies may unfairly incriminate a compound or overestimate or underestimate the risk of exposure in humans receiving pharmacological doses. For example, during development of omalizumab (XOLAIR®), a recombinant humanized anti-IgE monoclonal antibody that neutralizes circulating IgE, thrombocytopenia was identified and reproduced in preclinical safety studies in monkeys receiving the drug at 5–27 times the maximum clinical dose.¹ The thrombocytopenia observed in the monkeys was never severe enough to produce clinical hemorrhage. No effect on circulating platelet numbers was identified in clinical studies. After a temporary hold on development, it was determined that the observation was likely species-specific and platelet counts in further clinical trials were intensely monitored. To date, this has not materialized as an adverse effect in humans.³⁷

Conversely, preclinical testing can fail to predict toxicity at intended exposure. An example of this problem occurred when a low incidence (<0.3%) of agranulocytosis was detected in the late phase of clinical trials with prinomide, a pyrroleprionitrile, non-steroidal anti-inflammatory drug, resulting in immediate termination of further development of the drug.²⁶ No effect on hematopoiesis or circulating numbers of cells was evident in the preclinical studies (rodents, dog, monkey) conducted before these adverse reports. The unique difference between the individuals enrolled in the late phase studies who developed agranulocytosis and those in early phase clinical trials and animal models was that the late phase patients were experiencing active inflammatory conditions such as chronic arthritis. Because the metabolism of xenobiotics by myeloperoxidase has been associated with the formation of reactive metabolites capable of affecting normal cell function, further investigative work using the active metabolite of prinomide was conducted.²⁶ Mass spectroscopy revealed that the reactive metabolite was 1,4-benzoquinone, a compound implicated in myelotoxicity associated with benzene. The authors concluded that these reactive metabolites, produced under conditions of increased hydrogen peroxide (e.g. active inflammation) might explain the agranulocytosis identified in the late phase studies.

When test species have different abilities to detoxify by conjugation or reductive properties compared to humans, the toxic potential of a new chemical entity may not be identified until the first human studies are conducted. For example, a potential new drug administered to healthy adults unexpectedly produced methemoglobinemia during dose escalation studies (L.G. O'Rourke, personal observation). Again, full review of all preclinical data failed to predict this occurrence.

These examples are presented to first illustrate that classical animal models and preclinical studies, despite their rigors, may fail to predict life threatening adverse effects on the hematopoietic system in the intended species, or may overestimate risk of exposure. They also serve as reminders of the importance of understanding both species differences as well as similarities. Clinical safety studies function as a second line of defense, where discrepancies from preclinical studies usually can be detected. However, when toxicity occurs only in certain individuals within the human population, it may be difficult to determine if the responsible mechanism is due to a property of the drug or to the unique biology of an individual. Depending upon the incidence of this idiosyncrasy within the test population, this may not be detected in the pre-marketing phase of development. Many case reports in the literature present other instances of drug-induced hematotoxicity that were either not predicted in preclinical safety evaluation studies or were not identified until enough individuals were exposed post-marketing to detect an adverse effect.

INVESTIGATIVE WORK

Identification of cytopenia in individual or treatment group data needs to be addressed immediately. It is important to review all clinical observations, treatments, and handling to differentiate a direct compound-related effect on the hematopoietic system from a secondary effect such as blood loss (e.g. excess phlebotomy; gastrointestinal bleeding) or illness. Actions that may follow identification of a cytopenia in a safety study include:

- no change in protocol
- temporary suspension of dosing
- dose reduction
- cessation of dosing and initiation of a monitoring period
- humane termination

or a combination of the above. Obviously, if dosing is suspended and the findings resolve only to return when dosing is reinstated, the compound or its metabolites are clearly implicated.

It may be difficult to determine if an effect on the hematopoietic system is idiosyncratic when the incidence of an adverse finding is extremely low and is only found in one species. When a hematotoxicologic finding is identified in a preclinical study, it must be taken seriously and critically assessed. Depending upon the seriousness of the finding and the mechanism(s) involved, redesign of the molecule or termination of a project may ensue. On the other hand, when findings are limited to one species they may be interpreted as species-specific. In these instances, and depending upon the seriousness of the finding, there may be minimal impact on further development of the compound, although all clinical trials should be carefully monitored.

In addition to the *in vitro* and *in vivo* testing, efforts to develop animal models to test basic hypotheses con-

tinue. It is, however, challenging to identify animal models because reactions to xenobiotics in animals are as idiosyncratic as they are in humans.

CONCLUSIONS

Preclinical safety evaluation studies in animals and clinical safety evaluation studies in humans are designed to rigorously test new chemical entities for evidence of toxicity based on their relative risk/benefit. Because the number of animals exposed to a new chemical entity is relatively small, an adverse effect on the hematopoietic system must always be fully evaluated. When evidence of hematotoxicity occurs in human studies, either pre- or post-marketing, both in vivo and in vitro studies specifically designed to define the mechanism(s) are indicated. At present, many of these mechanisms remain a mystery. As the tools of pharmacogenomics and pharmacogenetics are advanced, our abilities to examine and understand interspecies drug-, class- and organ-specific pathways underlying toxicity, and to explore portions of the genome that may detect potential unanticipated impacts will improve, further enhancing our ability to select useful new xenobiotics that are not associated with hematotoxicity.

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Preclinical Evaluation of Compound-Related Alterations in Hemostasis

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Routine Laboratory Testing in Preclinical Studies
Investigation of Compound-Related Increases in PT or aPTT
Investigation of Compound-Related Clinical Signs of Altered Hemostasis
Biomarker Development for New Anticoagulant Therapies

Ex Vivo Experiments (New Anticoagulant Development)
Effect of Thromboplastin on Absolute Prothrombin Time
Conclusions

Acronyms and Abbreviations

aPTT, activated partial thromboplastin time; DIC, disseminated intravascular coagulopathy; FDA, Food and Drug Administration; FDP, fibrin degradation product; FX:C, Factor X clotting activity; ISI, international sensitivity index; PT, prothrombin time.

Hemostasis is a fundamental defense mechanism common to all vertebrates. It involves a delicately balanced process of blood clot (thrombus) formation that impedes blood loss from damaged blood vessels and thrombus dissolution (i.e. fibrinolysis, see Chapter 84). Species-specific differences in hemostasis do exist, so that direct translation of human-based assays can be problematic. Preclinical evaluation of compound-associated alteration in hemostasis is a prerequisite for new compound registration. Inadequate efficacy or poor safety margins are the two main reasons for compound attrition and are key to the success or failure of new compounds that are either purposefully designed to alter coagulation processes or inadvertently perturb hemostasis. This chapter will provide an overview of routine and non-routine laboratory testing requirements of blood coagulation among species important in designing preclinical trials and interpreting compound-related hematotoxicity, and in biomarker development during development of new anticoagulant therapies.

ROUTINE LABORATORY TESTING IN PRECLINICAL STUDIES

Harmonization of animal clinical pathology testing requirements for toxicity and safety studies was achieved by the Joint Scientific Committee for International Harmonization of Clinical Pathology Testing in 1996.⁸ Prothrombin time (PT), activated partial thromboplastin time (aPTT), and platelet count were the minimum recommended laboratory tests for hemostasis in small animals (mouse or rat) and large animals (dog or monkey).²

Clinical signs provide additional information to categorize acquired abnormalities in hemostasis. Clinical signs of primary hemostatic defects (failure of platelet plug formation) typically consist of bleeding from mucosal surfaces, cutaneous bruising, and hemorrhage following trauma or surgery. The pathways involved in secondary hemostasis comprise a cascade of enzymatic reactions that result in a stabilized fibrin clot. Clinical signs associated with defects in secondary hemostasis

include hemorrhage into joints, chest, or abdomen and subcutaneous or intramuscular hematoma formation. Excessive bleeding from sites of trauma or surgery are seen with secondary hemostatic defects as well as primary defects. Excessive bleeding following blood sample collection is often the first evidence of compound-related hemostatic defects in preclinical trials.

Changes in the intrinsic (contact system) coagulation pathway are routinely monitored using the aPTT, whereas changes in the tissue factor pathway (extrinsic pathway) are assessed by increases or decreases in PT. Both assays are assessed and expressed as the time to clot formation after addition of an exogenous clot activating agent. PT and aPTT are advantageous in that these assays are routine. Testing reagents are also standardized and instrumentation has undergone rigorous scrutiny to pass Food and Drug Administration (FDA) requirements because of their routine usage in clinical diagnostic laboratories. Reference intervals for PT and aPTT among species are variable, and experimental studies suggest that there is a marked difference in the rate of thrombin generation and fibrin formation among species.³ Use of non-species-specific laboratory reagents and instrumentation optimized for human testing may contribute to these differences. Therefore, it is important to generate species-specific reference intervals that are characterized by both reagent and instrumentation, and to select reagents and instrumentation that are compatible with the species required for preclinical examination.

Assessment of plasma fibrinogen is often included in assessment of compound-related effects on hemostasis as well as in general veterinary medicine. Decreases in fibrinogen, especially when accompanied by decreased platelet count, can be indicative of consumption of

coagulation factors associated with disseminated intravascular coagulopathy (DIC). In contrast, an increase in fibrinogenesis is suggested by an elevation in plasma fibrinogen levels. Hyperfibrinogenemia can be associated with infectious or inflammatory conditions including those associated with compound administration.⁴ Although effects on PT, aPTT, platelet count, and fibrinogen are valuable in screening for compound-related hematotoxicity, they are usually inadequate to provide a mechanistic explanation, particularly for unexpected compound-related toxicities.

INVESTIGATION OF COMPOUND-RELATED INCREASES IN PT OR APTT

Evidence of compound-related prolonged clotting times with or without decreases in fibrinogen requires investigation to determine the possible mechanism of action (Table 15.1). The most likely causes are specific or non-specific inhibitors of procoagulant plasma proteins in the coagulation cascade or consumption of these factors. The source of acquired alterations in hemostasis could arise from several compound-related issues. The liver is a common target for compound-related toxicity. Most coagulation factor proteins are synthesized in the liver. Therefore, hepatotoxicity can result in a decreased synthesis of coagulation proteins, and the resulting deficit is responsible for the prolongation of clotting times. If this is the case, decreases in other serum proteins such as total protein and albumin may be present, markers of hepatotoxicity such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, and/or bilirubin

TABLE 15.1 Useful Assays or Techniques to Determine the Mechanism of Action of Prolonged PT and/or aPTT in Preclinical Studies

Assay or Technique	Possible Compound-related Effect
Fibrinogen	↓ – Decreased production or consumption (possible DIC) ↑ – Inflammation
Hepatotoxicity screening assays	Serum/plasma chemistry assays (↑ALT, AST, alkaline phosphatase, bilirubin, GGT) Histopathology
Vitamin K plasma levels	Decreased absorption Decreased production by microflora
Coagulation factor-specific activity assay	Acquired compound-related factor deficiencies often affect multiple factors Single factor deficiencies can occur particularly for known anticoagulant compounds (i.e. anti-FXa inhibitors) ↓Factor II, VII, IX, and X for vitamin K-dependent processes
Vitamin K supplementation	Reversal of vitamin K-dependent toxicity
DIC panel	PT, aPTT, platelet count, FDP, D-dimer

are frequently elevated, and histologic evidence of liver pathology is also present.

Another possible explanation of decreased coagulation proteins is a compound-related effect on vitamin K-dependent proteins. Vitamin K is a fat-soluble vitamin that is either consumed in the diet or synthesized by bacterial microflora in the ileum and colon.⁷ Vitamin K-dependent coagulopathies are rarely observed in short-term preclinical studies because liver stores of vitamin K are adequate for several days. However, in studies exceeding two weeks duration, inflammatory or toxic disorders may lead to a relative or absolute deficiency of vitamin K. Compound-related vitamin K deficiencies are most frequently associated with:

1. studies conducted in neonatal animals due to decreased protein synthesis in the immature liver
2. studies using broad-spectrum antibiotics which decrease the bacterial flora in the intestines
3. studies that impact the absorption of fat soluble vitamins usually due to gastrointestinal, hepatic, renal or pancreatic disorders
4. studies using known antagonists of vitamin K such as warfarin.⁶

Factors II, VII, IX, and X are vitamin K dependent.

Several laboratory techniques can be used to identify vitamin K deficiency. Assessment of plasma vitamin K levels is possible but requires access to expensive and time-consuming mass spectrometry equipment. More commonly the quantitation of specific coagulation factors is based on the ability of test plasma to correct the clotting time of human plasma from an individual with an inherited deficiency of a single clotting factor. To accommodate for species differences in coagulation factors it has been suggested that a species- and reagent-specific standard curve be constructed using a pool of healthy individuals from the representative species. However, human-deficient plasma has adequate homology for diagnostic interpretations.³ Decreases in factor activity assays for vitamin K-dependent factors II, VII, IX, and X with no effect on factors that are vitamin K-independent, such as factor VIII, strongly suggest a vitamin K deficiency or antagonism. Reversal of these effects in animals given vitamin K supplementation further corroborates the finding.

If absolute factor activities are required, chromogenic assays should be used in lieu of factor activity assays. In these assays, prothrombin is activated by specific cleavage with the enzyme Ecarin, which is extracted from the venom of the *Echis carinatus* viper. Interspecies homology for the complete prothrombin molecule has been estimated at only 41%. However, there is no variance in the 10 glutamate residue required for vitamin K-dependent carboxylation.³ The prothrombin chromogenic assay has been shown to be effective in detecting prothrombin deficiency in a dog with vitamin K deficiency. This finding suggests that the assay could be useful in determination of compound-related effects on vitamin K.

Compound-related production of disseminated intravascular coagulopathy (DIC) can also be responsi-

ble for increases in PT and aPTT in preclinical studies (see Chapter 88). This condition is frequently accompanied by oozing of blood from venipuncture sites, hematomas, and hemorrhage. Coagulation factor proteins are consumed by an activation of the coagulation system and the fibrinolytic system is usually simultaneously activated. The underlying initiators of compound-related DIC can be diverse including systemic inflammation, uncontrolled immune-mediated cellular destruction (e.g. hemolysis), metabolic acidosis, and hepatosplenic disease. Diagnostic testing for DIC in preclinical studies typically includes PT, aPTT, platelet count, antithrombin III (ATIII) quantitation, and fibrin degradation products (FDPs) so that effects on accelerated coagulation and fibrinolysis are evaluated (see Chapter 138). Early assessments of FDPs used a rather non-specific precipitation of the protein fragments with protamine sulfate. These assays were useful in animal studies since they are not antibody dependent. More recently, D-dimers have been used to diagnose DIC in animals.⁶ D-dimers result only from the degradation of the stabilized fibrin clot. Therefore, the D-dimer assay is more specific for active coagulation and fibrinolysis and has proven to be useful in compound-related DIC observed in dogs and monkeys (KA Criswell, personal observation).

INVESTIGATION OF COMPOUND-RELATED CLINICAL SIGNS OF ALTERED HEMOSTASIS

In some preclinical studies clinical signs of bleeding or bruising may occur without effects on PT, aPTT, or other clotting assays (Table 15.2). The most frequent

TABLE 15.2 Useful Assays to Determine the Type of Hemostatic Alteration in Preclinical Studies

Initial Assay or Observation	Expanded Observation and/or Testing
Observation of clinical signs	Bleeding Hematomas Oozing of blood following collection of blood samples
Platelet count (decreased)	Histologic and/or cytologic evaluation of bone marrow for cellular depression Platelet lifespan (flow cytometry) Megakaryocyte ploidy (flow cytometry)
Platelet count (normal or increased)	Bleeding time (standardized template) Platelet activation (flow cytometry) Platelet aggregation (platelet-rich plasma or whole blood)

toxicity is a compound-related effect on platelet number or function. Drug-induced suppression of thrombopoiesis is a commonly reported syndrome for chemotherapeutic agents, estrogenic compounds, sulfadiazines, quinidines, and non-steroidal anti-inflammatory drugs (see Chapter 16). Platelet lifespan can be measured in most species by injecting sulfo-NHS-biotin and using flow cytometry to assess the time required to completely lose the biotinylated platelet label.⁵ Megakaryocyte ploidy can also be determined by flow cytometry.¹ Average megakaryocyte ploidy is 16N in nearly all species. Increased megakaryocyte ploidy in the presence of a decreased platelet count in preclinical trials suggests compound-related increased platelet turnover. Determining that reversal of bone marrow toxicity and depression of platelet counts occurs with withdrawal of compound administration is an important component of determining compound safety. Reversal studies should be a minimum of two weeks in length but are preferably four weeks in length to allow adequate time for bone marrow regeneration.

If clinical signs are apparent in the absence of effects on clotting factors or platelet count, effects on platelet function or effects on vasculature should be considered. In preclinical studies, functional assessment of vasculature defects can be problematic and identification of histologic abnormalities is often determined in lieu of functional assays. Functional platelet defects are more readily determined by laboratory assays (see Chapter 142). Bleeding time measurements can be rapidly and easily performed. Unfortunately, standardization of the bleeding time technique can be difficult in animals and may complicate interpretation of results. Variation of skin thickness in dogs and the site of bleeding time assessment in large or small animals can be particularly problematic. A spring-loaded device (Simplat II® or Surgicutt®) has been used to successfully standardize the bleeding time in the buccal mucosa of dogs, the ear tip of rats, and the forearm of monkeys (KA Criswell, personal observation). For rats, the pediatric version of the Surgicutt instrument should be used.

Low platelet counts may also be attributed to platelet activation and subsequent increased clearance of platelets. Platelet activation may be assessed by flow cytometry using a fluorescent marker for P-selectin (see Chapter 142). This technique requires very low volumes of blood so it is ideal for small animals, including mice. Urinary assessment of thromboxane A₂ metabolites can also provide an indirect assessment of platelet activation.¹ Platelet aggregation is the most frequently used methodology to assess platelet function (see Chapter 142). In contrast to the use of citrate for platelet aggregation assays in human, dog, or monkey samples, heparinized blood may be needed to adequately potentiate platelet aggregation results in rodents.¹ The most critical component of platelet function assays is appropriate sample collection. Platelet function assays should not be conducted when difficult sample collection is noted or any evidence of sample clotting is apparent on blood smears.

BIOMARKER DEVELOPMENT FOR NEW ANTICOAGULANT THERAPIES

Specific inhibitors of the coagulation pathway are actively being explored as new candidates for anticoagulant therapy. One of the documented areas is the utilization of biomarkers to measure activated coagulation factor X (FXa) to predict the clinical outcomes of FXa inhibitor compounds. FXa occupies a pivotal position within the coagulation cascade and is an attractive candidate as a target for coagulation intervention. This enzyme links the intrinsic and extrinsic coagulation pathways and is the rate-limiting step in thrombin formation. Rivaroxaban is an oral direct inhibitor of FXa. In a rat venous model of inferior vena cava thrombosis, Rivaroxaban produced a dose-dependent inhibition of FXa and an increase in PT. However, in a rabbit model, Rivaroxaban induced a 92% inhibition of FXa but only a 1.2-fold increase in PT. This demonstrates a marked species-specific variation in monitoring the newer anticoagulants with standard coagulation tests.

Rarely is a single biomarker considered definitive for evaluation of safety or efficacy. The drug development approach for Otamixaban and DU-176b incorporated a series of clotting assays and assays to measure the effects on thrombus formation.⁴ For Otamixaban, *in vitro* coagulation parameters were assessed for their ability to produce a doubling of PT and aPTT. This testing allowed a rank ordering of anticoagulant effect per species with rabbit > human > monkey > rat > dog. Additionally, aPTT appeared to be the more sensitive biomarker in all species. Multiple pharmacological models of thrombosis in rats, dogs, and pigs were also conducted with Otamixaban. In rats, thrombus mass was markedly reduced by nearly 95% with a corresponding increase in aPTT of 2.5-fold and PT of 1.6-fold.⁴ In contrast, intravenous administration of 1, 5, or 15 µg/mL of Otamixaban in the pig model effectively eliminated coronary flow reserves related to this stenosis model at the middle and high dose. PT was also prolonged at the middle and high dose, but aPTT was only prolonged at the high dose.

Ex Vivo Experiments (New Anticoagulant Development)

Development of new anticoagulants provides a unique opportunity of assessing drug efficacy *ex vivo* before conducting preclinical studies. This is important in planning preclinical studies because factor X concentrations vary between species and the level of drug-induced FXa inhibition produced is also variable. In typical studies, human or animal plasma is spiked with various concentrations of test compound. Chromogenic anti-FXa assays and factor X activity, using the factor X clotting (FX:C) assay can be used to determine efficacy of the drug. The FX:C assay provides several unique features that may make it a valuable biomarker for monitoring FXa inhibitor therapy:

1. The assay provides a rapid, reliable assessment of drug concentration and the percent inhibition of FXa achieved during drug inhibitor administration.
2. The assay can be performed on a high throughput automated system that is available in most hospital or veterinary coagulation laboratories.
3. Individual factor X concentrations range from 60% to 150% among human or preclinical subjects. This fairly high level of inter-subject variability suggests that a standard dose of drug may have a substantially different effect on total Factor X inhibition. The FX:C assay defines baseline factor X activity and thereby allows continued dosing to achieve a targeted factor X concentration.
4. Literature is available concerning factor X concentrations and bleeding history in patients with factor X deficiency, so minimally there is some understanding that correlates the impact of reductions in FX:C evaluations and bleeding potential in humans and animals.³

By determining the actual concentration of functional factor X remaining, individuals conducting pre-clinical or clinical trials have increased confidence in the administration of new FXa inhibitors.

As with other coagulation biomarkers used for monitoring FXa inhibition, it was not immediately clear whether the FX:C assay was applicable in multiple species. Ex vivo experiments allowed this evaluation. To provide effective anticoagulant activity, a 30% reduction in FX:C activity was predicted to be a minimal requirement. Table 15.3 shows the intended concentrations of a FXa inhibitor in each species, the resulting FX:C activity, and percent inhibition achieved.

Concentrations selected provided assessment of drug concentrations that induced FXa inhibition of approximately 20% to >90%, showing that the targeted range could be predicted and achieved in all species but the

TABLE 15.3 Factor X Activity and Percent Inhibition in Plasma Samples Containing Increasing Concentrations of the FXa Inhibitor^a

Species	Intended Drug Concentration (µg/mL)	FX:C Activity ^b (%)	Inhibition ^c (%)
Human	0	106.1 ± 1.9	NA
	0.2	64.3 ± 1.6	39.4
	0.6	32.2 ± 1.0	69.7
	1.2	16.5 ± 0.7	84.4
	1.8	10.0 ± 0.5	90.6
	6.0	2.3 ± 0.2	97.8
Dog	0	143.0 ± 4.5	NA
	0.4	112.9 ± 8.6	21.0
	2.0	42.4 ± 4.3	70.3
	8.0	11.1 ± 1.4	92.2
	15.0	5.4 ± 0.8	96.2
	Rat	0	84.8 ± 2.8
1.0		52.6 ± 1.8	38.0
4.0		26.2 ± 0.9	69.1
12.0		11.8 ± 0.6	86.0
24.0		6.7 ± 0.3	92.1

BLQ, below limits of quantification; NA, not applicable.
^aSamples spiked with a FXa inhibitor in vitro.
^bMean ± SD of 10 samples/concentration.
^cCalculated from species-specific control value.

TABLE 15.4 In Vitro Effect of an Experimental FXa Inhibitor on Absolute Prothrombin Time in Human, Dog, and Rat Plasma using Rabbit Brain Thromboplastin of Variable Potency (Defined By ISI Rating)

Concentration of FXa inhibitor (µg/mL)	0.98 ISI	1.24 ISI	1.55 ISI	2.21 ISI
PT (s) – Human Plasma				
0	11.4 ± 0.11	13.4 ± 0.17 ^a	12.9 ± 0.13 ^a	10.9 ± 0.14
0.2	18.3 ± 0.37	23.8 ± 0.50 ^a	23.4 ± 0.44 ^a	16.8 ± 0.43 ^a
0.6	30.8 ± 0.76	37.3 ± 0.86 ^a	39.9 ± 0.70 ^a	27.0 ± 0.93 ^a
1.2	47.0 ± 1.98	51.4 ± 1.51 ^a	58.9 ± 1.08 ^a	38.2 ± 1.39 ^a
1.8	61.2 ± 2.04	61.8 ± 1.58	74.8 ± 1.54 ^a	48.3 ± 2.08 ^a
6.0	133.0 ± 3.82	111.4 ± 3.17 ^a	152.3 ± 3.08 ^a	94.8 ± 4.12 ^a
PT (s) – Dog Plasma				
0	7.8 ± 0.14	8.4 ± 0.07	7.1 ± 0.07	6.7 ± 0.06
0.4	11.0 ± 0.26	11.0 ± 0.13	10.5 ± 0.19	10.2 ± 0.15
2.0	17.6 ± 0.42	16.6 ± 0.23 ^a	17.9 ± 0.41	15.0 ± 0.31 ^a
8.0	32.0 ± 0.98	27.6 ± 0.49 ^a	33.6 ± 0.92	27.1 ± 0.63 ^a
15.0	45.4 ± 1.52	36.5 ± 0.73 ^a	46.7 ± 1.35	36.8 ± 0.88 ^a
PT (s) – Rat Plasma				
0	9.1 ± 0.05	15.1 ± 0.07 ^a	17.1 ± 0.15 ^a	13.1 ± 0.07 ^a
1.0	13.3 ± 0.06	20.7 ± 0.13 ^a	31.3 ± 0.27 ^a	23.2 ± 0.19 ^a
4.0	20.1 ± 0.29	30.8 ± 0.23 ^a	51.8 ± 0.52 ^a	36.9 ± 0.52 ^a
12.0	31.1 ± 0.72	46.2 ± 0.49 ^a	83.6 ± 0.84 ^a	56.2 ± 0.84 ^a
24.0	42.7 ± 1.09	60.6 ± 0.73 ^a	109.4 ± 0.79 ^a	75.4 ± 1.60 ^a

^aSignificantly different from 0.98 ISI thromboplastin values at the 5 % level as determined by a t-test.

drug concentrations required to produce similar levels of FXa inhibition across species were markedly different. The FX:C assay helped determine drug concentration required for complete inhibition of FXa in these species and the relative bleeding risk associated with a range of factor X concentrations.

Effect of Thromboplastin on Absolute Prothrombin Time

Typically, the higher the international sensitivity index (ISI) value for thromboplastin, the less sensitive the reagent and the longer the PT produced. The most commonly used thromboplastin reagents for PT evaluation are either rabbit brain thromboplastin (variable ISI values depending on manufacturer) or human recombinant thromboplastin (typical ISI of 1.0). To more fully evaluate the effect of FXa inhibitors, PT was evaluated using rabbit brain thromboplastin with ISI values of 1.24, 1.55, and 2.21 and a human recombinant thromboplastin (0.98 ISI). The source and sensitivity of thromboplastin used in the assay affected the absolute PT value in all species, demonstrating the need to standardize this reagent in preclinical assessment and to be cognizant of the impact in clinical trials or in post-marketing where reagents are less likely to be standardized (Table 15.4).

CONCLUSIONS

Understanding compound-related effects of hemostasis continues to be an important aspect of marketing safe compounds and developing new anticoagulant therapies. Further, understanding of species-specific differences in hemostatic processes and interactions in assays typically optimized for human samples is critical in translating preclinical study data for the accurate prediction of safety and efficacy in humans.

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Drug-Induced Blood Cell Disorders

DOUGLAS J. WEISS

Mechanisms of Drug Toxicity
 Evaluation of Suspected Adverse Drug Reactions
 Adverse Drug Reactions in Dogs
 Antineoplastics
 Estrogen
 Anti-inflammatory
 Phenylbutazone/meclofenamic acid
 Carprofen
 Azathioprine
 Naproxen
 Antibacterials
 Sulfonamides
 Chloramphenicol
 Cephalosporins
 Anticonvulsants
 Phenobarbital/Primidone
 Phenytoin

Antiparasitics
 Cardiovascular
 Other Drugs
 Adverse Drug Reactions in Cats
 Antineoplastics
 Acetaminophen/Aspirin (see Chapter 36)
 Benzocaine/Cetacaine/Propofol (see Chapter 36)
 Phenazopyridine/DL-dmethionine (see Chapter 36)
 Azathioprine
 Propylthiouracil/Methimazole
 Griseofulvin
 Albendazole
 Azidothymidine
 Adverse Drug Reactions in Horses
 Drug-induced Immune-mediated Hemolytic Anemia
 Drug-induced Hemolysis
 Heparin

Acronyms and Abbreviations

ADR, adverse drug reaction; FDA, Food and Drug Administration; IMHA, immune-mediated hemolytic anemia; RBC, red blood cell.

A growing number of therapeutic drugs have been incriminated in adverse drug reactions (ADRs) associated with the hematologic system, with the greatest number being reported in dogs and cats.^{39,64,69} No attempt has been made to standardize the definition of drug-induced cytopenias in animals or to quantify the severity of these reactions. Standardized definitions of human drug-induced hematologic dyscrasias and evaluation of their severity have been described.⁸ This document defines cutoff values for neutropenia, leukopenia, thrombocytopenia, and anemia. It also defines the likelihood that a drug caused a hematologic dyscrasia as suggestive, compatible, incompatible, or inconclusive based on assessment of the time course between the beginning of exposure to the drug and discovery of the hemato-

logic dyscrasia. Neutropenia is defined as $<1,500$ neutrophils/ μL .⁸

Neutropenia is defined as compatible with an ADR if it is discovered during drug treatment. Neutropenia is considered inconclusive if it is discovered within 1 month after stopping exposure or more than 1 month after exposure if no leukocyte count was done in the interim. An increase in neutrophil count to $>1,500/\mu\text{L}$ within 1 month after stopping drug treatment is also suggestive of an ADR. Thrombocytopenia is defined as $<100,000$ platelets/ μL .^{8,70} Occurrence of thrombocytopenia within 1 month after initiation of drug treatment or remission within 3 weeks after stopping treatment is suggestive of an ADR.

Although this document cannot be directly adapted to drug-induced hematologic dyscrasias in animals, it

provides a general framework for evaluation of drug-induced hematologic dyscrasias in domestic animal species.

MECHANISMS OF DRUG TOXICITY

Drug-induced blood dyscrasias have been categorized as Type A or Type B reactions.⁴² Type A ADR are dose-dependent while Type B ADR are idiosyncratic reactions that are unrelated to the drug's pharmacologic effects.⁴² Idiosyncratic drug reactions are the most challenging to define and may involve a variety of mechanisms.^{14,42,69} Unique genetic or acquired susceptibility of the individual is usually involved. Genetic susceptibility may involve mutations that alter drug metabolism or induce an immune response to a drug or its metabolites.⁶¹ Acquired susceptibility may occur because of hepatic or renal disease leading to altered metabolism or excretion of a drug or its metabolites.⁶¹

The site and mechanism by which a drug acts is important in determining the clinical syndrome and prognosis; however, some drugs act at multiple sites or by several mechanisms making this determination difficult (Table 16.1). Destruction of cells in the peripheral blood results in rapid onset of cytopenias. Because the bone marrow can compensate by increasing production, decreased cell lifespan in the blood can be compensated and cell numbers may return to normal despite continued administration of the drug. Red blood cells (RBCs) are particularly sensitive to oxidative injury.²¹ Many drugs have oxidant properties or are metabolized to oxidants.²¹ These drugs tend to be dose-dependent because tissue injury is largely dependent on the degree of oxidative injury (see Chapter 36). Drugs associated with oxidative injury in dogs include acetaminophen, aspirin, menadione, methylene blue, benzocaine, phenacetin, and phenylhydrazine.^{15,21,30} Cats are more sensitive to the oxidant effects of many drugs because of the presence of eight reactive sulfhydryl groups per molecule compared to two in most other species.²¹ Drugs associated with Heinz body anemia in cats include acetaminophen, aspirin, cetacaine, methylene blue, phenazopyridine, DL-methionine, and propofol (see Chapter 36).^{15,21,30}

Rapidly proliferating progenitor and proliferative cells in bone marrow are susceptible to chemotherapeutic agents. Destruction of these cells results in predictable changes in the blood. Neutropenia occurs initially followed by thrombocytopenia.¹⁶ Anemia develops more slowly depending on the RBC lifespan of the particular species. Discontinuation of the drug usually results in hematologic recovery. Bone marrow aspiration smears in some affected animals may reveal toxic changes in precursor cells. One drug-associated change is the presence of multiple distinct cytoplasmic vacuoles that are most prominent in granulocyte precursors (Fig. 16.1).

Some drugs induce hematopoietic stem cell destruction or alter the hematopoietic microenvironment. The timing of development of cytopenias is more

TABLE 16.1 Drugs Suspected or Confirmed to Cause Hematologic Dyscrasias in Animals

Class and Drug	Species	Suspected Mechanism ^a
Antineoplastics		
Class effect	All	MS, D
Endocrine		
Estrogen	Dog	MS
Propylthiouracil	Cat	IM
Methimazole	Cat	IM
Anti-inflammatory		
Phenylbutazone	Dog, Cat	MS, IM
Meclofenamic acid	Dog	MS
Azathioprine	Dog, Cat	MS
Naproxen	Dog	BS
Antibacterial		
Chloramphenicol	Dog	D
Cephalosporin	Dog	IM, MS, D
Penicillin	Horse	IM
Sulfonamides	Dog	IM, MS
	Horse	IM
Antifungal		
Griseofulvin	Cat	MS
Antiviral		
Azidothymidine	Cat	MS
Anticonvulsants		
Phenobarbital/Primidone	Dog	MS
Phenytoin	Dog	MS
Antiparasitics		
Levamisole	Dog	MS
Albendazole	Dog, Cat	MS
Metronidazole	Dog	MS
Fenbendazole	Dog	MS
Cardiovascular		
Amiodarone	Dog	IM
Captopril	Dog	MS
Quinidine gluconate	Dog	Unknown
Analgesics		
Acetaminophen	Dog, Cat	O
Aspirin	Dog, Cat	O
Phenacetin	Dog	O
Anesthetics		
Benzocaine	Dog, Cat	O
Ceracaine	Cat	O
Propofol	Cat	O
Urinary acidifiers/antiseptics		
Methylene blue	Dog, Cat	O
Diphenylhydrazine	Dog	O
DL-Methionine	Cat	O
Phenazopyridine	Cat	O
Other drugs		
Human erythropoietin	Dog	IM
Mitotane	Dog	MS
Colchicine	Dog	MS
Phenothiazine	Horse	O
Dimethyl sulfoxide	Horse	O
Heparin	Horse	H

^aMS, bone marrow suppression; IM, immune-mediated blood cell destruction; BS, blood loss; D, dysmyelopoiesis; O, oxidant; H, hemolysis.

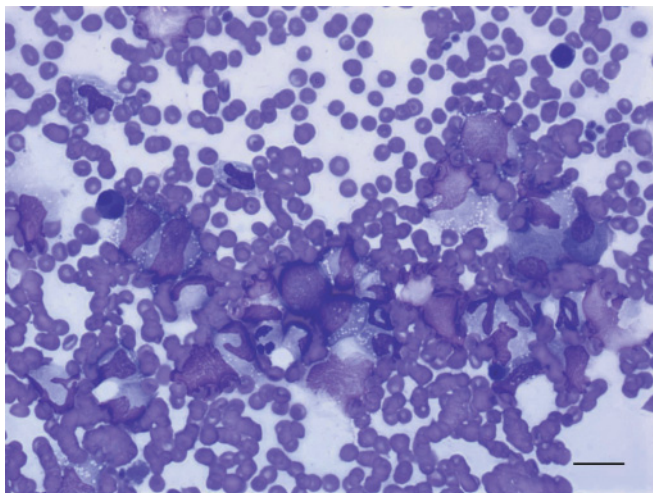


FIGURE 16.1 Bone marrow aspirate from a dog treated with cyclophosphamide. Note the presence of distinct cytoplasmic vacuolization in granulocyte precursors. Wright-Giemsa stain; bar, 20 μ m.

unpredictable and recovery is uncertain and delayed when it occurs. Endothelial cells of marrow capillaries and sinusoids are vulnerable to toxic injury (see Chapter 17). Vascular injury can be seen in bone marrow core biopsy sections as interstitial edema, hemorrhage, necrosis, myelofibrosis, or acute inflammation.⁶⁸

EVALUATION OF SUSPECTED ADVERSE DRUG REACTIONS

The mere temporal association of a drug treatment with a hematologic disorder does not in itself provide proof of an ADR. In each case other potential causes of the hematologic condition should be eliminated by evaluation the history, and clinicopathologic findings. When an ADR is suspected, the temporal relationship between onset of treatment and detection of the hematologic dyscrasia, previous exposure to the drug in question, and recovery from the hematologic dyscrasia after drug withdrawal should be documented.^{8,42} This information not only documents the likelihood of a cause and effect relationship but may suggest a possible mechanism for the drug toxicity. When multiple drugs have been administered before detection of hematologic dyscrasias, the Food and Drug Administration (FDA) database (<http://www.fda.gov/cvm/adetoc.htm>) can be reviewed or a literature search conducted to determine if other similar toxicities have been reported.⁴² The FDA database can also be used to report suspected toxicities. Usually this information combined with serial complete blood counts, tests for immune-mediated hematologic diseases, and bone marrow aspirates and core biopsy specimens are the only data available to link a drug to a clinical ADR. Other tests that might be done include evaluation of the hemolytic effects of the drug and evaluation of the suppressive effects of the drug, serum, or patient lymphocytes on blood cell

TABLE 16.2 Myelosuppressive Potential of Chemotherapeutic Drugs used in Dogs and Cats

High	Moderate	Low to None
Cyclophosphamide	Methotrexate	Corticosteroids
Cytosine arabinoside	Chlorambucil	L-Asparaginase
Doxorubicin	6-Mercaptopurine	Bleomycin
Vinblastine	Melphalan	
Hydroxyurea	5-Fluorouracil	
	Vincristine	

or bone marrow cytotoxicity or on in vitro bone marrow cell culture.^{24,40,50}

ADVERSE DRUG REACTIONS IN DOGS

Antineoplastics

Chemotherapeutic agents cause Type A ADR. Because of their high mitotic rate, progenitor and proliferative cells in bone marrow are predisposed to injury by chemotherapeutic drugs. The myelosuppressive potential of chemotherapeutic agents varies (Table 16.2).¹⁶

Cytopenias are the most frequent chemotherapeutic toxicity identified and often necessitate temporary or permanent discontinuation of treatment. Bone marrow suppression follows a predictable course based primarily on the half-life of cells in the blood. Neutropenia occurs 5–7 days after myelosuppression followed by thrombocytopenia 9–14 days after myelosuppression.¹⁶ Anemia as a direct result of chemotherapy is usually not seen because of the comparatively long half-life of RBCs.¹⁶ Neutrophil and platelet counts usually return to baseline values within 72–96 hours after discontinuing therapy.¹⁶

Beyond destruction of hematopoietic cells, chemotherapeutic drugs have few adverse effects on the hematopoietic system. Multifocal areas of coagulation-type necrosis have been reported in association with administration of cyclophosphamide and vincristine.⁶⁵ An increase in dysplastic hematopoietic cells and atypical mitotic figures (i.e. secondary dysmyelopoiesis) is a frequent finding and should not be confused with a myelodysplastic syndrome (see Chapter 66).^{1,67} Doxorubicin administration to dogs has been associated with poikilocytosis including the presence of ovalocytes, echinocytes, schistocytes, and keratocytes.⁵ Doxorubicin is thought to bind to spectrin, a major cell membrane structural protein.⁵

Estrogen

Dog and ferret bone marrow is highly susceptible to estrogen-induced suppression, and both dose-dependent (Type A ADR) and idiosyncratic (Type B ADR) reactions have been documented in dogs (aplastic anemia is discussed in Chapter 39).^{34,55,69} In dogs, both a single large dose of estradiol and therapeutic doses of estradiol

as well as therapeutic doses of diethylstilbestrol result in hematologic dyscrasias. Therapeutic doses of estradiol or diethylstilbestrol, used in treatment of mammary tumors or prostatic hyperplasia, consistently cause a transient decrease in platelet count and neutrophilia 10–20 days after administration.²² Administration of a single large dose of estradiol to dogs results in consistent hematologic dyscrasias. Leukocytosis, thrombocytopenia, and mild but progressive nonregenerative anemia develop in the first 3 weeks after injection of 1–3 mg/kg body mass of estradiol. Pancytopenia with bone marrow aplasia develops between 3 and 4 weeks after injection and most dogs begin to recover after 4 weeks. Although the mechanism is complex and poorly understood, serum inhibitors of hematopoiesis produced by T cells have been identified.²³

Individual dogs appear to be uniquely sensitive to the effects of estrogen and develop severe aplastic anemia. Dogs given repeated therapeutic doses of estradiol or dogs having elevated endogenous estrogen levels resulting from cystic ovarian follicles, ovarian granulosa cell tumors, or Sertoli cell tumors may develop hematologic dyscrasias. Dogs with endogenous estrogen toxicity typically are pancytopenic but some are bicytopenic.^{34,55,69} The bone marrow is typically aplastic.^{2,22,25} This appears to be the result of hematopoietic stem cell destruction. The prognosis for these dogs is poor but recovery after weeks to months of supportive care has been documented.⁶⁹ Myelofibrosis, bone marrow necrosis, and dysmyelopoiesis have been observed in dogs given a therapeutic doses of estradiol propionate.^{34,69}

Anti-Inflammatory

Phenylbutazone/Meclofenamic Acid

Many cases of phenylbutazone-associated hematologic dyscrasias have been reported and one case of meclofenamic acid-associated aplastic anemia has been reported. Phenylbutazone-associated neutropenia or pancytopenia occurs sporadically in dogs.^{15,54} Several types of Type B ADR have been observed. One is a transient agranulocytosis that typically occurs within 2 weeks after starting treatment. Affected dogs frequently recover promptly when the drug is discontinued. This condition is most probably immune-mediated but inhibition of bone marrow cell division or DNA synthesis is also a possible explanation. In another type of hematologic dyscrasia, dogs develop aplastic anemia months or years after initiation of treatment, presumably as a result of bone marrow toxicity.^{63,69} In the experience of the author, the prognosis for recovery is poor. Additionally, phenylbutazone therapy has been associated with myelonecrosis and myelofibrosis.^{63,69}

Carprofen

Coagulation-type myelonecrosis has been reported in bone marrow from several dogs treated with therapeutic doses of carprofen.⁶⁵ Another dog was reported to have a neutrophilic dermatitis, vasculitis, immune-

mediated hemolytic anemia, and thrombocytopenia associated with carprofen administration.⁴³

Azathioprine

Neutropenia and thrombocytopenia or pancytopenia have been observed one to several months after initiation of treatment with immunosuppressive doses of azathioprine.^{36,52} Bone marrow is aplastic and some dogs have mild myelofibrosis. Peripheral blood cell numbers slowly return to normal once the drug is discontinued but complete recovery may be delayed.

Naproxen

Naproxen administration has been associated with blood loss anemia.^{20,27} Gastrointestinal erosions or ulcerations are suspected to be the cause of the blood loss. This may result from local irritation or inhibition of prostaglandin synthesis.

Antibacterials

Sulfonamides

Adverse drug events have been reported for sulfadiazine, sulfamethoxazole, and sulfadimethoxine.^{26,66,69} These drugs induce an idiosyncratic syndrome that includes neutropenia, thrombocytopenia, hemolytic anemia, fever, polyarthropathy, or hepatopathy.²⁶ Doberman pinschers may be at increased risk of sulfonamide hypersensitivity.²⁶ Others have reported sulfadiazine-associated aplastic anemia (Fig. 16.2).^{66,69} The mechanism appears to be immune-mediated and does not appear to be related to folate deficiency.^{26,60} Evidence of an immune-mediated etiology includes demonstration of drug-dependent anti-platelet antibodies and a positive direct Coombs' test.²⁶ In dogs

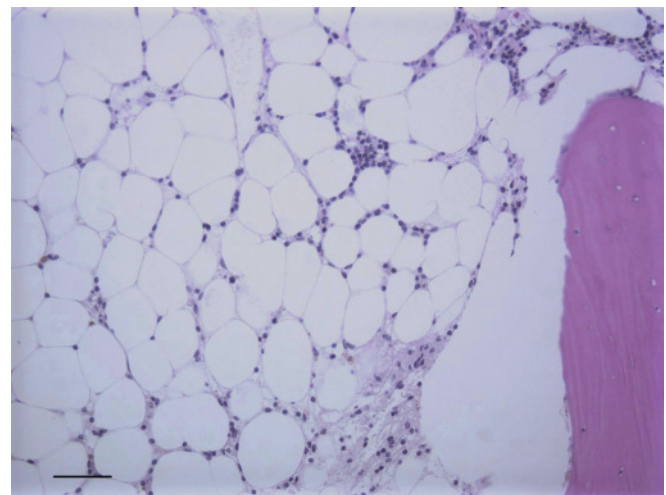


FIGURE 16.2 Bone marrow core biopsy from a dog treated with trimethoprim-sulfadiazine for 15 days. Note the presence of marrow aplasia. Cells present are mostly small lymphocytes. Hematoxylin & Eosin stain; bar, 200 μ m.

with sulfadiazine-induced aplastic anemia, pancytopenia usually occurs 10–14 days after initiation of treatment.^{25,66} The bone marrow is typically aplastic with the hematopoietic space replaced by adipose tissue. The hematologic dyscrasia usually resolves within 2 weeks after discontinuing treatment. In another study, recovery occurred in 90% of non-thrombocytopenic dogs but in only 63% of thrombocytopenic dogs.⁶⁰

Chloramphenicol

Severe aplastic anemia has been reported as an idiosyncratic reaction in humans treated with chloramphenicol but has not been reported in dogs given therapeutic doses.⁶⁴ One apparent idiosyncratic reaction was reported.³¹ This dog had a moderate non-regenerative anemia with large numbers of siderocytes and ringed sideroblasts. This response suggests a reversible dysmyelopoiesis perhaps secondary to drug-induced mitochondrial damage.

Cephalosporins

Certain cephalosporins have been associated with several types of hematologic dyscrasias.^{9,18} High-dose or prolonged administration of cephalosporins can induce agranulocytosis, thrombocytosis, and a positive direct Coombs' test.¹³ Approximately half of dogs given high doses of cefonicid or cefazedone intravenously developed pancytopenia within 6–10 weeks after initiation of treatment.^{9,18} The mechanism appears to be complex. In one study, the presence of anti-RBC, anti-neutrophil, and anti-platelet antibodies was consistent with immune-mediated destruction.⁹ Cephalosporins also induce alteration in the bone marrow. Granulocytic and erythroid maturation arrest have been reported in dogs treated with cephalosporins.¹⁸ The most prominent ultrastructural change in bone marrow was mitochondrial damage to hematopoietic and non-hematopoietic cells.¹⁸ The hematologic dyscrasia resolved rapidly after discontinuing the drug. Aplastic anemia associated with persistent suppression of hematopoietic stem cell activity also has been reported when dogs were administered high doses of cefazedone.¹³ Further, cefoperazone, cefamandole, and moxalactam inhibit synthesis of vitamin K-dependent clotting factors and can lead to hypoprothrombinemia and a resultant coagulopathy.¹³

Anticonvulsants

Phenobarbital/Primidone

Phenobarbital and primidone therapy have been associated with several types of hematologic dyscrasias in dogs. Because primidone is metabolized to phenobarbital, the mechanism of toxicity may be similar for both drugs.⁴¹ The mildest hematologic disorder is neutropenia and thrombocytopenia.^{37,59,65} The presence of granulocyte hyperplasia in bone marrow indicates that the neutropenia is due to destruction of mature granulo-

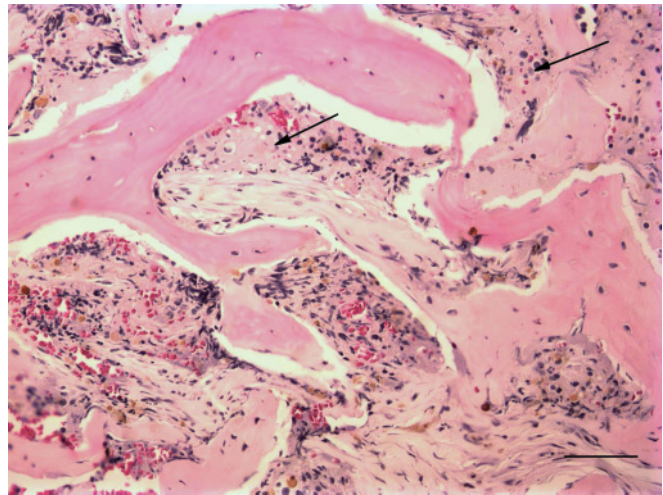


FIGURE 16.3 Bone marrow core biopsy from a dog treated with therapeutic doses of phenobarbital. Note the presence of coagulation-type necrosis (arrows) and myelofibrosis. Hematoxylin & Eosin stain; bar, 200 μ m.

cytes. A more frequent ADR is myelonecrosis or myelofibrosis (Fig. 16.3).^{65,71} Dogs with myelonecrosis are frequently bicytopenic or pancytopenic. Bone marrow is characterized by multifocal areas of coagulation-type necrosis with variable degrees of myelofibrosis present.⁷¹ Dogs with myelofibrosis, without concurrent necrosis, frequently have a severe non-regenerative anemia. The myelofibrosis may represent the chronic stage of myelonecrosis.

Phenytoin

Long-term administration of phenytoin to dogs results in RBC macrocytosis, neutropenia, thrombocytopenia, and neutrophil hypersegmentation.¹¹ These changes were thought to be due to folate deficiency; however, folate deficiency does not cause RBC macrocytosis in dogs.¹¹ One dog undergoing long-term phenytoin therapy developed a severe normocytic non-regenerative anemia associated with severe myelofibrosis in the bone marrow.⁷¹

Antiparasitics

Several antiparasitic drugs have been associated with Type B ADR. Levamisole-induced thrombocytopenia and direct Coombs' positive hemolytic anemia have been reported in several dogs.^{3,4} All dogs had hyperplastic bone marrow and recovered after discontinuing levamisole treatment. Albendazole toxicity has been documented in one dog.⁵⁷ The dog had pancytopenia and an aplastic bone marrow, but recovered within 1 week after discontinuing treatment. Three dogs treated with metronidazole had coagulation-type myelonecrosis.⁶⁵ All dogs had pancytopenia but recovered after discontinuation of treatment. Fenbendazole administration has been associated with bone marrow coagula-

tion-type necrosis.⁶⁵ One dog had pancytopenia after repeated weekly administration of the organic arsenical, thiacetarsamide for treatment of demodectic mange.⁶² The bone marrow was hypercellular and the dog recovered after discontinuation of treatment. However, the dog subsequently developed bone marrow RBC aplasia and died.

Cardiovascular

Several cardiovascular drugs have been associated with Type B ADR. Amiodarone is an antiarrhythmic drug that has been associated with hemolytic anemias and hepatopathies in dogs.^{12,35,38} Hematologic alterations were characterized by thrombocytopenia and positive direct Coombs' test results with or without the presence of anemia. Affected dogs recover when treatment is discontinued. One dog developed pancytopenia and aplastic bone marrow after 18 months of continuous administration of captopril for treatment of a heart murmur.³⁵ Withdrawal of the drug and treatment of the dog with human granulocyte-colony stimulating factor and erythropoietin resulted in prompt hematologic recovery. *In vitro* studies using canine bone marrow cell cultures indicate that the toxic effects of captopril are dependent on a reactive thiol group.²⁸ This thiol group reacts with copper to generate hydrogen peroxide which is toxic to canine neutrophil progenitor cells. One dog developed neutropenia and anemia after prolonged administration of quinidine gluconate.⁶⁹ Three days after discontinuing the drug the neutrophil count returned to normal and bone marrow cellularity was normal.

Others Drugs

Many dogs given human erythropoietin for a prolonged period of time develop antibodies to the recombinant protein.^{17,51} In addition to blocking the biologic effects of human erythropoietin, the antibody cross-neutralizes endogenous canine erythropoietin resulting in profound erythroid hypoplasia.^{17,51} The onset of the immune response is typically 2–3 months after initiation of treatment. Administration of recombinant canine erythropoietin to dogs suffering from recombinant human erythropoietin-associated red cell aplasia does not appear to be effective in resolving the anemia in dogs with blocking antibodies.⁵¹

Several dogs given mitotane or colchicine were reported to have coagulation-type myelonecrosis consistent with drug-induced bone marrow hematotoxicity.⁶⁵ Withdrawal of drug treatment resulted in hematologic recovery.

ADVERSE DRUG REACTIONS IN CATS

Antineoplastics

Neutropenia and thrombocytopenia are frequently dose-limiting factors in chemotherapy.¹⁶ As in other

species, rapidly proliferating progenitor and proliferative cells in bone marrow are susceptible to destruction by chemotherapeutic agents. Beyond cytopenias, dysplastic hematopoietic cells and atypical mitotic figures (i.e. secondary dysmyelopoiesis) are frequently found in bone marrow.⁶⁷ Doxorubicin administration to cats has been associated with poikilocytosis including the presence of ovalocytes, echinocytes, schistocytes, and keratocytes.⁴⁵

Azathioprine

Cats appear to be highly sensitive to azathioprine-induced bone marrow suppression. Of five cats given 2.2 mg/kg of azathioprine on alternate days, four developed multiple cytopenia.⁷ Azathioprine-induced bone marrow suppression in humans has been associated with homozygosity for the gene for thiopurine methyltransferase.⁷ This enzyme plays an important role in the catabolism of azathioprine.

Propylthiouracil/Methimazole

Severe Type B ADR, consisting of agranulocytosis and severe thrombocytopenia, has been reported in 8.6% of hyperthyroid cats treated with propylthiouracil and 3.8% of cats treated with methimazole.^{48,49} Mild hematologic alterations, including eosinophilia, lymphocytosis, and mild leukopenia, were reported in 16.4% of cats treated with methimazole.⁴⁹ Clinical signs develop within 1–2 months after initiation of treatment and include lethargy, weakness, anorexia, and platelet-related bleeding diathesis. Many affected cats were fluorescent antinuclear antibody test and direct Coombs' test positive, indicating that the hematologic dyscrasia was immune-mediated. These hematologic abnormalities resolved within 2 weeks after discontinuing methimazole treatment.

Griseofulvin

Griseofulvin is a fungistatic antibiotic used for treatment of mycotic diseases.³³ Neutropenia or panleukopenia occur as an idiosyncratic reaction to griseofulvin in cats.^{33,53} In most cases, this occurs several weeks after initiation of griseofulvin treatment.³³ The bone marrow is typically hypoplastic. The majority of cats recover after treatment is discontinued.

Albendazole

Albendazole toxicity has been reported in two cats.⁵⁷ Both animals were pancytopenic but had hypercellular bone marrow. Both cats had rapid hematologic recovery within 1 week after discontinuing treatment.

Azidothymidine

Azidothymidine is a dideoxynucleoside derivative reverse transcriptase inhibitor used for treatment of

feline immunodeficiency virus and feline leukemia virus infections. A transient or progressive nonregenerative anemia develops after several weeks of treatment.³² The anemia is primarily the result of bone marrow erythroid hypoplasia but Heinz body formation may also be a contributing factor.

ADVERSE DRUG REACTIONS IN HORSES

Drugs associated hematologic ADRs in horses include penicillin and trimethoprim-sulfamethoxazole-associated immune-mediated hemolytic anemias (IMHAs), heparin-associated thrombocytopenia and RBC agglutination, dimethyl sulfoxide-L-tryptophan-indole, and phenothiazine-induced hemolysis, and phenylbutazone-associated aplastic anemia.

Drug-induced Immune-mediated Hemolytic Anemia

Penicillin administration to horses has been associated with acute allergic reactions and with IMHA.^{10,56} These allergic reactions consist of acute anaphylaxis, collapse, shaking, urticaria, fever, leukopenia, eosinophilia, thrombocytopenia, and anemia, and are thought to be an adverse response to procaine.¹⁰ Penicillin and its metabolites bind firmly to the RBC membrane. These penicillin-protein complexes appear to frequently induce detectable antibody formation; however, the anti-penicillin antibodies tend to be IgM, are present in low titer, and do not cause hemolysis.^{10,56} Anti-penicillin antibodies, that induce immune-mediated hemolytic anemia, tend to be IgG.¹⁰

One horse developed IMHA during treatment with trimethoprim-sulfamethoxazole.⁵⁸ Prompt recovery after withdrawal of drug treatment incriminated the drug in the causation of the anemia.

Drug-induced Hemolysis

Phenothiazine poisoning has been associated with hemolytic anemia in a horse.⁶ Although this was thought to be an oxidant-induced hemolysis, few Heinz bodies were seen.

Oral or intravenous administration of L-tryptophan or indole to ponies results in acute hemolytic anemia and hemoglobinuria.^{46,47} Indole is the major metabolite of L-tryptophan that induces hemolysis. Indole has lipophilic properties and interacts with RBC membranes, perhaps explaining their hemolytic properties. However, some ponies treated with indole had Heinz bodies present in RBCs suggesting a possible Heinz body anemia.

Rapid intravenous administration of a 40% solution of dimethyl sulfoxide results in intravascular hemolysis in horses.² The hemolytic effects of dimethyl sulfoxide appear to be concentration dependent and administration of a more dilute solution significantly reduces or eliminates the hemolysis.² The mechanism of dimethyl

sulfoxide-induced hemolysis is uncertain but appears to involve potassium leakage from erythrocytes before hemolysis.

Heparin

Unfractionated heparin has been reported to cause marked thrombocytopenia as an idiosyncratic reaction in human patients within 4–14 days after initiation of treatment.²⁹ Heparin appears to exert a direct effect on platelets causing platelet activation and aggregation, and also induces anti-platelet antibodies. In horses, the most frequent complication associated with heparin administration is anemia.¹⁹ This is associated with RBC agglutination. Heparin may act as a hapten for anti-RBC antibodies.⁴⁴ Rapid increases in hematocrit occur after discontinuing heparin treatment. Because the increase in hematocrit exceeds the expected capacity of the bone marrow to replace erythrocytes, it is suspected that heparin causes *in vivo* agglutination of RBCs in the microvasculature.⁴⁴ Thrombocytopenia, fatal hemorrhage, and swelling at injection sites have also been reported. In humans, the thrombocytopenia is immune-mediated.

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Myelonecrosis and Acute Inflammation

DOUGLAS J. WEISS

Microvascular Injury

Myelonecrosis

Acute Inflammatory Response

Immune-mediated disease

Systemic lupus erythematosus

Infectious diseases

Drugs (see Chapter 16)

Hematopoietic Cell Injury

Heavy Metals

Lead

Mercury

Arsenic

Chemicals

Benzene

Chlorinated hydrocarbons

Drugs (see Chapter 16)

Toxins

Mycotoxins

Bracken fern

Infectious Agents (see Chapter 19)

Acronyms and Abbreviations

DDT, chlorophenothane; DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetic acid; IMHA, immune-mediated hemolytic anemia; ppm, parts per million; RBC, red blood cell.

As a rapidly proliferating tissue, bone marrow is sensitive to toxic injury. Sites most vulnerable to injury include progenitor and proliferating hematopoietic cells and the microvasculature. Microvascular injury results in altered vascular permeability and manifests as edema, hemorrhage, inflammation, or ischemia.⁴² Myelonecrosis occurs when ischemia is severe and in the chronic stage necrotic areas may become fibrotic.^{16,39}

Hematopoietic cell injury can result in hematopoietic cell death at various stages of maturation and arrested development with dysplastic changes.³² Hematopoietic cell destruction usually results in a hypocellular marrow. In the acute stages of hematopoietic injury, myelotoxic changes can be seen. These are best observed in the cytoplasm of metamyelocytes and band neutrophils. These cells have a foamy basophilic cytoplasm that results from the clumping of RNA. Some cells may contain Dohle bodies.³² Drugs toxic to bone marrow may induce distinct cytoplasmic vacuoles (Fig. 16.1, p. 100).³² Toxic changes in the nucleus are less frequent but consist of hypersegmentation or bizarre segmentation of neutrophils. Donut-shaped nuclei are abnormal in dogs and primates but are a normal finding in rodents. Arrested maturation frequently is characterized by cytopenias in the blood, a cellular bone marrow with

arrested development, and dysplastic changes in one or more cell lines. Dysplastic changes can be seen as nuclear or cytoplasmic maturation defects. Nuclear maturation defects result from interference with DNA synthesis and include megaloblastosis, large cell size, atypical mitotic figures, fragmented nuclei, and binucleation. Dysplastic cytoplasmic changes are most frequently seen in erythroid precursor cells where hemoglobin production is inhibited. Bone marrow is characterized by large numbers of small metarubricytes that frequently have indistinct cytoplasmic margins.

MICROVASCULAR INJURY

The bone marrow microvasculature is susceptible to toxic injury.³⁶ This is supported by experimental studies in which mouse bone marrow was evaluated after injection of endotoxin and rabbit bone marrow was evaluated after saponin injection.^{19,48} Early ultrastructural lesions in bone marrow are characterized by degeneration of sinusoidal endothelial cells. Later lesions consisted of dilated sinusoids, interstitial edema, hemorrhage, and necrosis. Chronic lesions consisted of myelofibrosis.

Similar lesions have been described in humans and a variety of animal species.^{4,10,23,36,44} Acute vascular

injury is accompanied by dilated sinusoids, interstitial edema, and hemorrhage.^{10,36} Acute inflammation is characterized by exudation of fibrin that may be accompanied by an infiltration of neutrophils.^{10,44} Vascular injury also results in varying degrees of ischemia. When severe, ischemia induces ischemic necrosis. Depending on which change is most prominent, the pathologic process may be defined as acute inflammation or as myelonecrosis.^{39,44}

Myelonecrosis

Myelonecrosis is usually associated with bone marrow ischemia.^{16,39} Ischemia can be enhanced if anemia, hypoxemia, or hypoperfusion is present. Acute severe hematopoietic cell injury can also result in myelonecrosis. Myelonecrosis is difficult to appreciate in bone marrow aspiration smears. The necrotic marrow smears unevenly, resulting in irregular elongated clumps of necrotic cells. Microscopically, the slides have a diffuse amorphous eosinophilic background and cells stain poorly. Many cells are lysed and in others nuclei appear smudged and the cytoplasm is vacuolated with indistinct borders. In histopathologic sections, myelonecrosis can appear as multifocal areas of coagulation-type necrosis or as individual cell necrosis (Fig. 17.1).³⁹ Coagulation-type necrosis appears as a central area of karyorrhectic and pyknotic cells enmeshed in an amorphous eosinophilic background material. Macrophages are frequently increased in number. Coagulation-type necrosis occurs frequently in dogs but is rarely seen in cats.⁴⁰ Individual cell necrosis is observed in approximately 8% of cat clinical bone marrow specimens and is characterized by large numbers of degenerating, frequently anuclear, hematopoietic cells and increased pink amorphous background material (Fig 17.2).⁴⁰ In the acute stage of myelonecrosis, necrosis is frequently accompanied by acute inflammation or hemorrhage, whereas in subacute myelonecrosis, necrosis is associated with phagocytic macrophages presumably removing the necrotic cells. In chronic myelonecrosis, reticulin or collagen myelofibrosis is frequently present.

Frequent causes of myelonecrosis in cats and dogs include immune-mediated hemolytic anemia (IMHA), sepsis, and leukemia/lymphoma. Other causes identified in dogs are idiopathic causes, systemic lupus erythematosus, disseminated intravascular coagulopathy, and drug treatments.^{39,41} Drugs associated with myelonecrosis include estradiol, phenobarbital, carprofen, mitotane, colchicine, metronidazole, fenbendazole, cephalosporins, and chemotherapeutic agents including cyclophosphamide and vincristine (see Chapter 16).

The mechanisms responsible for myelonecrosis have not been extensively investigated. In immune-mediated diseases and disseminated intravascular coagulopathy, thrombotic events may induce marrow ischemia. The necrosis associated with systemic lupus erythematosus in humans has been attributed to the presence of anti-phospholipid antibodies. In sepsis, endotoxin or other bacterial toxins may induce sinusoidal injury as well as being toxic to hematopoietic precursor cells. Leukemic

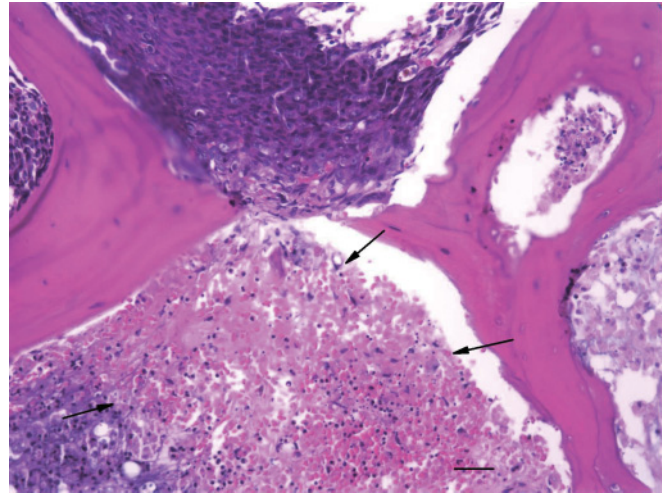


FIGURE 17.1 Coagulation necrosis (arrows) associated with a metastatic pancreatic carcinoma (top) in a bone marrow core biopsy specimen from a dog. Hematoxylin & Eosin stain; bar, 200 μ m.

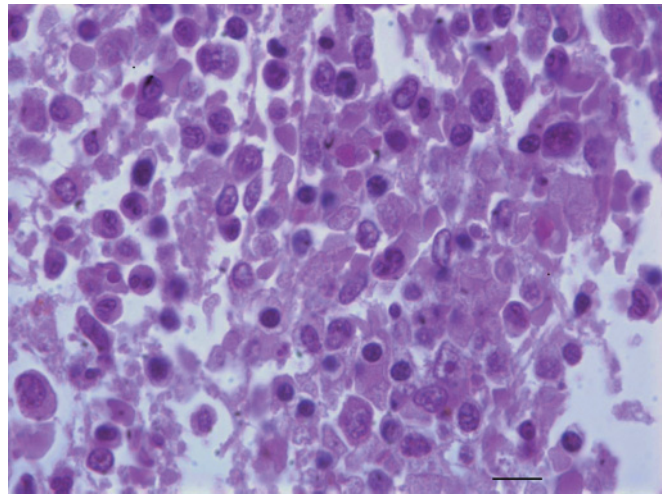


FIGURE 17.2 Individual cell necrosis in a core biopsy section from a cat with non-regenerative immune-mediated hemolytic anemia. Note the presence of many anuclear cells and cellular debris. Hematoxylin & Eosin stain; bar, 20 μ m.

cells in bone marrow frequently outgrow their blood supply resulting in myelonecrosis.

Dogs and cats with myelonecrosis have various combinations of non-regenerative anemia, leukopenia, and thrombocytopenia.^{39,41} In some instances the cytopenias may be the result of myelonecrosis whereas, in other animals, they are the result of a combination of the primary disease process and myelonecrosis. Treatment and prognosis for myelonecrosis should be based on the cause of the necrosis.^{39,41} In general, if the primary condition can be successfully treated, the prognosis for complete recovery is good.³⁹

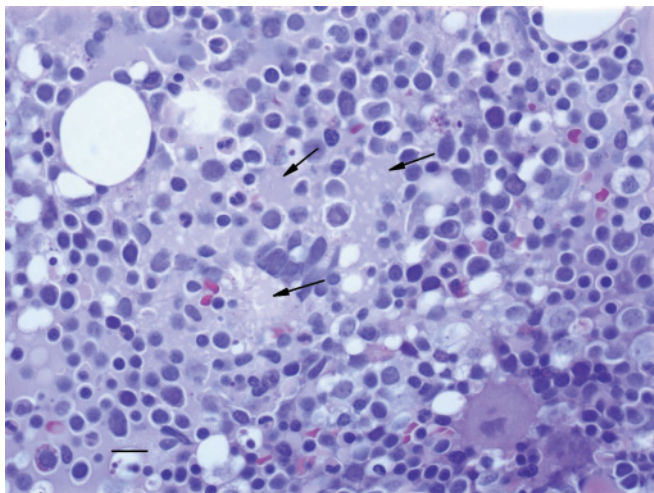


FIGURE 17.3 Interstitial edema (arrows) in a bone marrow core biopsy section from a dog with immune-mediated hemolytic anemia. Hematoxylin & Eosin stain; bar, 20 μ m.

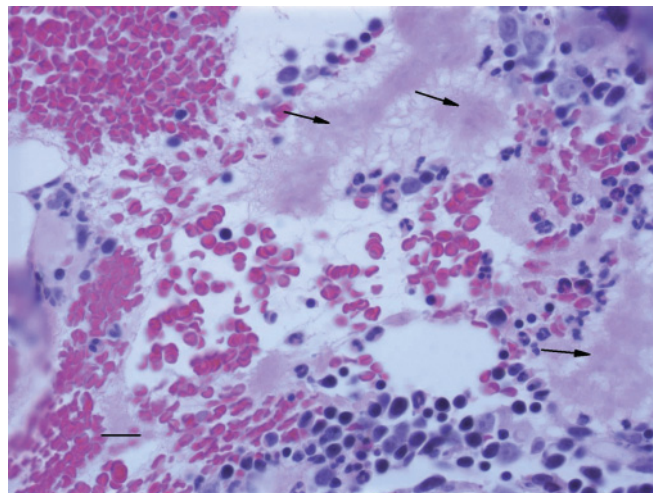


FIGURE 17.4 Fibrinous inflammation in a bone marrow core biopsy section from a dog with immune-mediated hemolytic anemia. Note the presence of fibrin (arrows), and hemorrhage. Hematoxylin & Eosin stain; bar, 20 μ m.

Acute Inflammatory Response

Acute inflammation in bone marrow, like myelonecrosis, is thought to result from acute injury to bone marrow sinusoids.¹⁰ These changes are not usually seen in bone marrow aspirates but are visible in bone marrow sections. Acute inflammation has been observed in approximately 3% of canine clinical bone marrow specimens.⁴² The initial lesion in bone marrow is altered vascular permeability. Histopathologic alterations include dilated or congested sinusoids, interstitial edema, and hemorrhage. Interstitial edema is seen as amorphous pink proteinaceous material between hematopoietic cells (Figs. 17.3 and 17.4).^{42,43} Pathologic hemorrhage must be differentiated from artifactual hemorrhage induced by the biopsy procedure. When the sample is obtained by needle biopsy, artifactual hemorrhage is frequently present on either end of the biopsy specimen and may be present on the edges of the specimen. Pathologic hemorrhage should be observed within the hematopoietic space away from the ends or edges of the specimen and should be accompanied by evidence of vascular alterations or interstitial edema. These alterations cannot be reliably evaluated in bone marrow specimens obtained post-mortem. Dilated sinusoids and altered permeability can occur as agonal changes or perhaps be induced by administration of euthanasia solution.

Microvascular injury can also result in exudation that is characterized by multifocal fibrin deposits or by multifocal infiltrates of neutrophils (Fig. 17.5).^{42,43} Fibrin deposits are particularly prominent in cat bone marrow.^{41,43} Fibrin appears as pink fibrillar material. The presence of fibrin can be confirmed by selective staining with Frazier-Lundrum stain.⁴⁴ Frequently, these fibrin deposits contain variable numbers of segmented neutrophils. In cases of sepsis, such as septicemia or endocarditis, discrete microabscesses may be

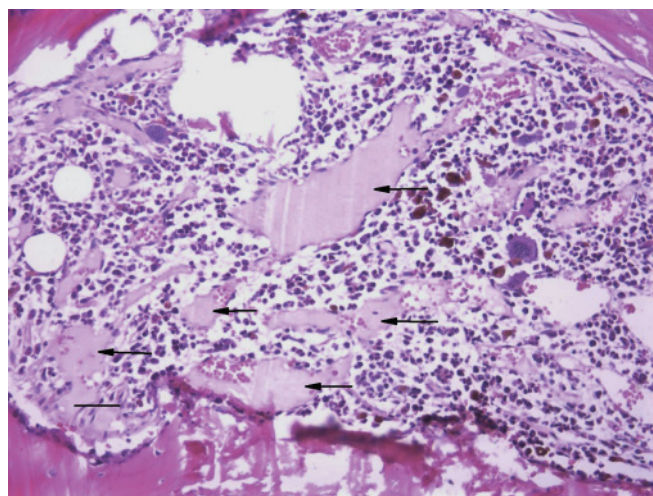


FIGURE 17.5 Sinusoidal dilation (arrows) in a bone marrow core biopsy section from a dog with bacterial sepsis. Also note the presence of large numbers of segmented neutrophils consistent with acute inflammation. Hematoxylin & Eosin stain; bar, 100 μ m.

present in bone marrow (Fig 17.4).⁴² These structures may represent septic thrombi.

Causes of acute inflammation in dog bone marrow include nonregenerative IMHA, systemic lupus erythematosus, and infectious diseases; in cat they include IMHA and infectious diseases.⁴¹⁻⁴³ Therefore, identifying acute inflammation in bone marrow should prompt a search for these conditions.

In horses and cattle, acute inflammatory responses in bone marrow have been associated with bacterial sepsis.⁴⁴ Acute inflammation has been identified in septicemic foals. Lesions are characterized by large multifocal infiltrates of neutrophils resembling microabscesses. Neutrophils in the lesions may appear degenerate and

the centers of the microabscesses may be necrotic. Other lesions included dilated sinusoids, hemorrhage, and fibrin deposition. Bacteria are usually not seen in bone marrow sections but can be cultured from bone marrow in some cases. Perivascular neutrophilic infiltrates in bone marrow were reported in a steer with chronic pneumonia caused by *Mannheimia haemolytica*.⁴⁴ Other cattle with a diagnosis of bacterial pneumonia and septicemia had fibrin deposits in bone marrow.

Eosinophilic inflammatory responses are less well defined. A cow and a sheep with fibrinous inflammation and large numbers of eosinophils in bone marrow have been described.⁴⁴ The cow had bacterial enteritis and the sheep had a systemic vasculitis of unknown origin.

HEMATOPOIETIC CELL INJURY

A variety of heavy metals, chemicals, drugs (see Chapter 16), toxins, and infectious agents (see Chapter 19) injure hematopoietic precursor cells. Hematopoietic cell injury frequently results in hypocellular marrow and if all cells lines are affected can result in aplastic anemia. Some drugs and toxins induce dysplastic changes that can result in peripheral cytopenias with normocellular or hypercellular bone marrows.

Heavy Metals

Several heavy metals, including lead, mercury, and arsenic cause bone marrow alterations in addition to significant toxic changes in other tissues.

Lead

Lead poisoning occurs in domestic species, including dogs, cats, and cattle, as well as in waterfowl and some exotic species. The source of lead exposure varies by species. In dogs and cats, lead-containing paints; in cattle, discarded batteries and industrial contamination of pastures; and in waterfowl, lead shot are the major sources of exposure.^{1,21,24} In dogs and cats, anemia can be associated with chronic lead poisoning. In dogs, basophilic stippling and metarubricytosis have been described as classical features. However, these changes appear to be infrequent findings. Collection of blood in ethylenediaminetetraacetic acid (EDTA) may cause the stippling to disappear.⁴⁵ The pathogenesis of the anemia involves a shortened red blood cell (RBC) lifespan and dyserythropoiesis. Lead may shorten RBC survival by inhibiting membrane ATPase.¹⁵ Lead poisoning in dogs is also associated with an increase in the myeloid-to-erythroid ratio and dyserythropoiesis. Lead interferes with several enzymes in the heme synthesis pathway and inhibits pyrimidine 5'-nucleotidase that is involved in depolymerization of reticulocyte ribosomal RNA. Resultant retention of RNA in reticulocytes may be responsible for the basophilic stippling seen in some animals.

Mercury

Mercury has hematotoxic and immunotoxic effects.⁵ Fish, waterfowl, and shore birds are at greatest risk of exposure to environmental mercury contaminants.³⁷ Exposure of egrets or ducks to low dose methylmercury results in anemia with increased bone marrow cellularity.³⁷ This suggests a hemolytic anemia. Hemolysis may be due to the capacity of mercury to inhibit several RBC enzymes including glutathione reductase, glutathione-6-phosphate dehydrogenase, and acetylcholinesterase. Mercury also induces idiosyncratic immune-mediated hematologic disorders. Exposure of egrets to high dose methylmercury results in bone marrow hypoplasia and depletion of lymphocytes in lymph nodes and thymus.³⁷ Mercury has been shown to be cytotoxic to hematopoietic cells and to cause dysmyelopoiesis in laboratory animals.⁶

Arsenic

Arsenic poisoning causes both hemolytic anemia and dyserythropoiesis, and has genotoxic effects. Arsenic in the form of arsine gas is a potent hemolytic agent; however, the main source of exposure is to humans in industrial settings.¹⁸ In the environment, arsenic exists in inorganic forms and the main source of exposure is through contaminated drinking water.² Severe poisoning in humans results in marked anemia, and in some cases neutropenia and thrombocytopenia.⁸ Arsenic poisoning causes marked dyserythropoiesis characterized by karyorrhexis, megaloblastosis, and multinuclearity. This is thought to result from a direct toxic effect on DNA synthesis that results in altered nuclear division.^{8,22} Results of several studies conclude that inorganic arsenic has genotoxic effects in a variety of laboratory animal species.² These conclusions are based on results of studies evaluating the presence of micronuclei and DNA strand breaks in bone marrow.

Chemicals

A large variety of chemicals have the potential to alter hematopoiesis depending on the dose and duration of exposure. A few examples of chemicals with relatively high hematotoxic potential will be discussed here.

Benzene

Benzene and its metabolites are highly toxic to the hematopoietic system.^{31,47} Benzene is widely used as a solvent in the manufacture of drugs, chemicals, dyes, and explosives. Because of work-related exposure, benzene toxicity is primarily a problem in humans. Benzene has both cytotoxic and genotoxic effects on the hematopoietic system. A variety of hematologic dyscrasias have been observed including hemolytic anemia, bone marrow hyperplasia, leukopenia, dysmyelopoiesis, aplastic anemia, myelodysplastic syndromes, leukemia, and lymphoma.^{31,47} Concentrations as low as 100 parts per million (ppm) have been associated with

leukopenias in approximately one-third of exposed workers.¹⁴

Experimental inhalation or injection of benzene produces aplastic anemia in rabbits and mice.¹³ The aplasia is preceded by dysmyelopoiesis. Despite the typical aplastic marrow and pancytopenia, stem cell numbers in marrow are normal suggesting that committed progenitor cells rather than stem cells are damaged by benzene. However, benzene-treated lethally irradiated marrow is not reconstituted by transplantation of normal marrow. This suggests that benzene damages the marrow microenvironment.

Chlorinated Hydrocarbons

A variety of chlorinated hydrocarbons are toxic to bone marrow and have the potential to cause clinical disease. These drugs include carbon tetrachloride, hexachlorophene, lindane, chlordane, chlorobenzene, chlorophenothane (DDT), chlorinated dioxins, pentachlorophenol, and trichloroethylene. Some of these compounds, including DDT, lindane (i.e. gamma-benzene hexachloride), and pentachlorophenol, are derivatives of benzene. Chlordane, DDT, lindane, and pentachlorophenol have been implicated as causes of aplastic anemia in humans and trichloroethylene has been implicated as a cause of aplastic anemia in cattle (see Chapter 39).^{9,30,33} Although the pathogenesis of the injury is uncertain, these compounds have multiple injurious effects on hematopoietic tissue. Chlorinated hydrocarbons appear to be toxic to primitive hematopoietic cells. Exposure of mice to various doses of lindane transiently reduced the number of pleuripotent stem cells and progenitor cells.¹⁷ In addition, residual long term progenitor cell depletion has been demonstrated.¹⁷ Dysmyelopoiesis is also a frequent finding associated with chlorinated hydrocarbon toxicity. Many chlorinated hydrocarbons also have genotoxic effects. The genotoxic potential of 1,1-dichloroethane has been evaluated in Swiss-Webster mice by evaluation of mitotic index, micronuclei, and chromosomal aberrations.²⁸ A dose-related increase in the number of chromosomal aberrations and micronuclei formation was observed.

Many fatal cases of aplastic anemia in cattle in Minnesota in the 1950s were traced to feeding of trichloroethylene-extracted soybean oil meal.³⁰ Although no longer used for this purpose, trichloroethylene is widely used as a solvent.¹¹ In experimental studies, tetrachloroethylene-treated mice had a reduction in erythroid and granulocytic progenitor cells but no decrease in pleuripotent stem cells following inhalation exposure for 7.5 weeks.³⁴ Trichloroethylene has been shown to inhibit delta-aminolevulinic acid dehydratase, an enzyme involved in heme synthesis, in rats.¹¹ Trichloroethylene also appears to have genotoxic effects.³⁸

Chlorinated dioxins cause anemia and progressive leukopenia in a variety of species including chickens, primates, and rats.²⁶ Chickens appear to be highly sensitive and develop clinical signs after ingestion of feeds containing fats industrially contaminated with dibenzo-

p-dioxin. Administration of this material to chickens, primates, and rats resulted in hypoplasia of bone marrow and lymphoid tissue in all three species.²⁶

Toxins

Toxins that are directly cytotoxic to hematopoietic precursor cells include mycotoxins and bracken fern. Myelotoxicity results in hypoplastic or aplastic changes in bone marrow.

Mycotoxins

A variety of mycotoxins produce aplastic anemia in animals and people. Mycotoxin exposure occurs with ingestion of food contaminated with fungal organisms. For animals, this is frequently the result of ingestion of grain that has been allowed to become damp during storage. Aplastic anemia has been documented in horses and cattle ingesting feed contaminated with *Stachybotrys alternans*. Many other mycotoxins, including T-2 mycotoxin, trichothecene mycotoxin, trichothecene vomitoxin, ochratoxin, and zearalenone, produce toxicity in humans.⁷ These toxins appear to be directly cytotoxic to a variety of cells including hematopoietic precursor cells and lymphocytes. As a result, many mycotoxins cause bone marrow hypoplasia/aplasia and lymphocyte depletion.¹² Depletion of stem cells and granulocyte-macrophage progenitor cells was detected when mice were exposed to ochratoxin.³ A marked increase in apoptotic hematopoietic cells was detected in bone marrow after injection of T-2 mycotoxin into mice.³⁵ The effect of mycotoxins on bone marrow and the immune system appears to be amplified by exposure to lipopolysaccharide. Genotoxic effects of some mycotoxins have also been documented in mouse studies.

Bracken Fern

Bracken fern (*Pteridium aquilinum*) is one of the most widely distributed plants on earth. Animals repeatedly ingesting bracken fern develop aplastic anemia (see Chapter 39).²⁰ Cattle, horses, sheep, and pigs may be affected. The major toxin in bracken is ptaquiloside.⁴⁶ Ptaquiloside is a thiaminase.⁴⁶ After repeated exposure and thiamine depletion, cattle tend to develop aplastic anemia. This results in anemia, hemorrhage, and increased susceptibility to infection. Other species develop neurological signs due to polioencephalomalacia.²⁰

Chronic enzootic hematuria in cattle has also been linked to development of urinary bladder neoplasia after prolonged ingestion of bracken fern.²⁵ Metabolites of ptaquiloside are carcinogenic and induce bladder and intestinal carcinomas in cattle and other farm animals as well as in laboratory animals.^{25,29} Lymphocytic leukemia and pulmonary tumors have also been detected in mice exposed to ptaquiloside.²⁷

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Chronic Inflammation and Secondary Myelofibrosis

DOUGLAS J. WEISS

Chronic Inflammation

Lymphocytosis

Plasmacytosis

Lymphoid Aggregates

Macrophage Proliferation

Reactive

Hemophagocytic syndrome (see Chapter 45)

Malignant histiocytosis (see Chapter 73)

Acute monocytic and myelomonocytic leukemia
(see Chapter 67)

Chronic myelomonocytic leukemia (see Chapter
65)

Lipid storage disorders

Granulomatous Inflammation

Secondary Myelofibrosis

Osteosclerosis

Serous Atrophy of Fat/Gelatinous Transformation

Acronyms and Abbreviations

FeLV, feline leukemia virus; IMHA, immune-mediated hemolytic anemia; PRCA, pure red cell aplasia.

Chronic bone marrow injury can occur as a sequela of acute bone marrow injury or after prolonged or recurrent exposure to a toxic agent.²² Acute injuries that incite a chronic bone marrow response include myelonecrosis and acute inflammatory responses (see Chapter 17).^{34,38} These chronic responses in bone marrow include chronic inflammatory responses (e.g. lymphocytosis, plasmacytosis, macrophage proliferation, and granulomatous inflammation), secondary myelofibrosis, osteosclerosis, osteomyelitis, and serous atrophy of fat/gelatinous transformation.^{2,10,31,42} As with acute bone marrow injury, many of these lesions are difficult or impossible to detect in bone marrow aspirates. Therefore it is essential to evaluate both bone marrow aspirates and histopathologic sections to detect chronic bone marrow injury.

CHRONIC INFLAMMATION

Lymphocytosis

Lymphocyte numbers in bone marrow vary with species and age. Rodents have relatively large numbers of lymphocytes, whereas domestic animals, avian species, and primates typically have less than 10% lymphocytes in cellular bone marrow specimens.^{12,30} However, reference intervals reported for the percentage of lymphocytes in cat bone marrow vary with most indicating that up to 15% should be considered normal.^{19,35} In

healthy animals, most lymphocytes in bone marrow are small lymphocytes.¹² Benign lymphocytosis is the most frequent type of chronic inflammation in cats but occurs infrequently in dogs.^{36,37} In a retrospective study of 203 consecutive feline bone marrow reports, lymphocytosis (defined as lymphocytes >15% of all nucleated cells) was observed in 15.6% of the specimens.³⁶ In some cats, lymphocyte numbers exceeded 50% of all nucleated cells in bone marrow.³⁵ Greater than 80% of cats with lymphocytosis had a diagnosis of non-regenerative immune-mediated hemolytic anemia (IMHA) or pure red cell aplasia (PRCA).^{35,36} Differentiating benign lymphocytosis from chronic lymphocytic leukemia can be problematic. Lymphocyte distribution and phenotype may be useful in differentiating these conditions. Benign lymphocytes are frequently arranged in small multifocal aggregates in bone marrow, whereas, in chronic lymphocytic leukemia, they are diffusely distributed throughout the marrow.^{35,43} Immunophenotyping of a few cases has revealed that most lymphocytes in reactive lymphocytosis are B cells, whereas malignant lymphocytes in chronic lymphocytic leukemia are usually T cells.^{35,43}

Unlike the cat, only 1% of canine clinical bone marrow specimens had lymphocytosis.³⁷ These cases were associated with immune-mediated diseases including IMHA and PRCA. Therefore, at least in the Northern USA, bone marrow lymphocytosis in both cats and dogs correlates with immune-mediated hematologic diseases.

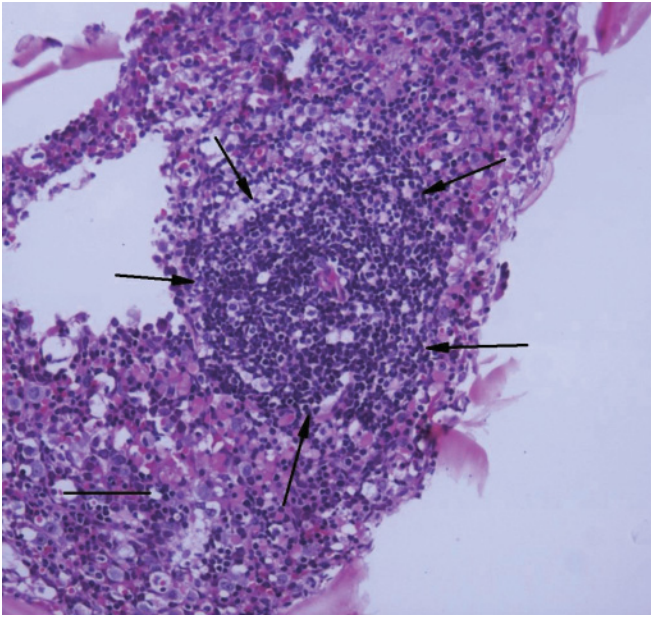


FIGURE 18.1 Lymphoid aggregate (arrows) in a bone marrow core biopsy section from a cat with nonregenerative immune-mediated hemolytic anemia. Hematoxylin & Eosin stain; bar, 200 μm .

Plasmacytosis

Plasmacytosis is usually evaluated subjectively in bone marrow aspirates or core biopsy specimens because of their low numbers and uneven distribution.¹² Plasmacytosis has been observed in approximately 10% of canine clinical bone marrow specimens and in 6% of cat bone marrow specimens.^{36,37} Plasmacytosis in cats has been associated with IMHA, PRCA, immune-mediated thrombocytopenia, and feline infectious peritonitis. Plasmacytosis in dog bone marrow has been associated with IMHA, PRCA, and immune-mediated thrombocytopenia. Other causes of plasmacytosis that should be considered include *Ehrlichia* and *Leishmania* infections.

Lymphoid Aggregates

In a review of 17 species of mammals, lymphoid aggregates were identified only in humans and cats.^{13,14} Lymphoid aggregates are small clusters of mature small lymphocytes with no evidence of organized structure (Fig. 18.1). However, results of large retrospective studies indicate that lymphoid aggregates occur infrequently in cat and dog bone marrow core biopsy specimens.^{36,37} When present, lymphoid aggregates correlate with the presence of immune-mediated hematologic diseases or other chronic systemic immunologic stimulation.^{35,41}

Macrophage Proliferation

The presence of increased numbers of macrophages in bone marrow is associated with a variety of disorders.

Macrophages can range from relatively immature cells with minimal phagocytosis to mature cells that have a foamy cytoplasm or contain cells, cytoplasmic debris, or hemosiderin. Four general types of mononuclear phagocyte proliferations can be observed in bone marrow. Differentiating these types can be problematic and may require the use of flow cytometry or immunophenotyping.³³ The four types of proliferation include reactive macrophage proliferation, reactive hemophagocytic syndrome^{16,39} (see Chapter 45), lipid storage diseases, and malignant proliferations of monocytes (acute monocytic or myelomonocytic leukemia, chronic myelomonocytic leukemia; see Chapters 65 and 67), and dendritic cells (malignant histiocytosis, see Chapter 73). Reactive macrophage proliferations are macrophages responding to a need to remove necrotic debris or to infectious agents. Increased necrotic debris is present in the subacute stages of myelonecrosis and in conditions of ineffective hematopoiesis.³⁵ Ineffective hematopoiesis is present in instances of myelodysplastic syndromes, immune-mediated hematologic diseases, and in some leukemias. In each of these conditions the presence of increased numbers of macrophages is commonplace.

A variety of infectious agents cause reactive macrophage proliferations in bone marrow. These vary with the species and geographic distribution of infectious organisms. In dogs, leishmaniasis, parvovirus infection, and deep fungal infections have been associated with macrophage proliferations (see Chapter 19).^{4,11} In leishmaniasis and deep fungal infections macrophages appear to be part of an inflammatory response in that organisms are found in the marrow in these conditions. Additionally, the presence of increased numbers of neutrophils is consistent with a diagnosis of a pyogranulomatous inflammation. In parvovirus infection, macrophages may be reacting to the need to remove necrotic cells.

Lysosomal storage disorders are a group of genetic deficiencies in lysosomal enzymes classified by the substance that accumulates in lysosomes. This results in the presence of large numbers of foamy macrophages in many tissues including the bone marrow. These cells tend to have a characteristic appearance.

Granulomatous Inflammation

Granulomatous inflammation can consist of a mixed infiltrate of macrophages, giant cells, small lymphocytes, or plasma cells, or can consist of distinct granulomas. Granulomatous inflammation has been associated with disseminated histoplasmosis in dogs and cats and *Mycobacterium avium* subsp. *avium* infection in dogs (see Chapter 19).^{7,8,18}

Granulomas are distinct compact aggregates of macrophages (Fig 18.2). These may contain epithelioid macrophages that have a large amount of pale cytoplasm and elongated nuclei with a dispersed chromatin pattern. Epithelioid cells may fuse to form giant cells. Other cells associated with the granuloma may include lymphocytes, plasma cells, and neutrophils. Granulomas

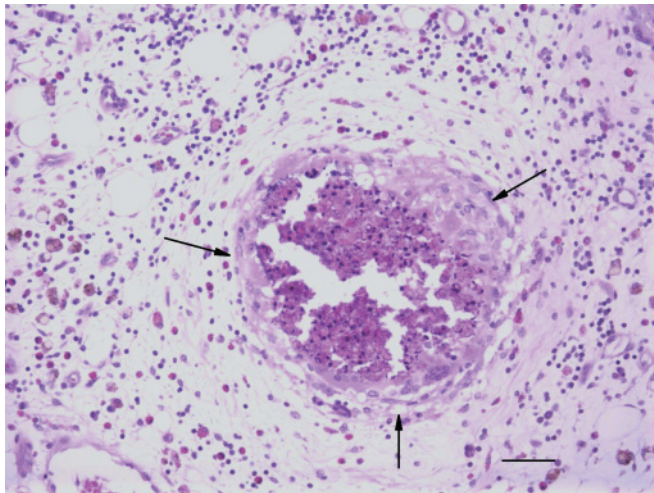


FIGURE 18.2 Granuloma (arrows) in a bone marrow core biopsy section from a dog with a disseminated fungal infection. Hematoxylin & Eosin stain; bar, 200 μ m.

are infrequently seen in dog and cat bone marrow.^{36,37} Granulomas have been associated with systemic fungal infections in dogs and a horse with an idiopathic systemic granulomatous disease.⁴² Lipid granulomas have been observed in a cow with serous atrophy of fat.⁴² Lipid granulomas consist of aggregates of lipid-laden macrophages. Other cells that may be present in lipid granulomas include lymphocytes, plasma cells, epithelioid cells, and giant cells.¹⁰ In humans, bone marrow granulomas have been associated with a variety of drug hypersensitivities. These drugs include phenytoin, procainamide, phenylbutazone, chlorpropamide, sulfasalazine, ibuprofen, indometacin, allopurinol, and carbamazepine.²

SECONDARY MYELOFIBROSIS

Secondary myelofibrosis is a bone marrow disorder characterized by proliferation of fibroblasts or collagen or reticulin fibers in the hematopoietic space (Fig. 18.3).^{1,2} Secondary myelofibrosis must be differentiated from idiopathic myelofibrosis that is a rare chronic myeloproliferative disease (see Chapter 65). Secondary myelofibrosis occurs relatively frequently and is associated with moderate to severe non-regenerative anemia and less frequently with thrombocytopenia or leukopenia.³² Collagen and fibroblastic myelofibrosis has been observed in 4.2% of canine clinical bone marrow specimens and in 9% of feline clinical bone marrow specimens evaluated at a veterinary teaching hospital.^{36,37} The distribution of fibrosis in marrow sections varies. Reticulin fibers are most frequently diffusely distributed in the marrow but in some early lesions can be focally distributed around blood vessels. Fibrosis can be random and multifocal, diffuse, or paratrabeular. When paratrabeular myelofibrosis is detected, renal osteodystrophy and primary hyperparathyroidism should be considered (Fig. 18.4).² The fibrosis is thought

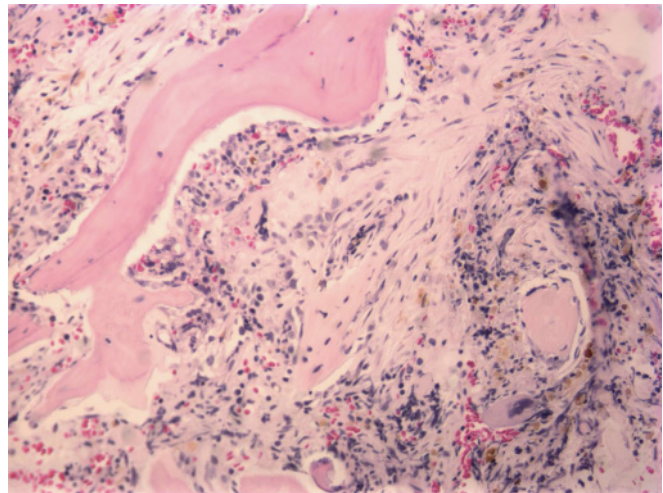


FIGURE 18.3 Secondary myelofibrosis in a bone marrow core biopsy section from a dog. Hematoxylin & Eosin stain.

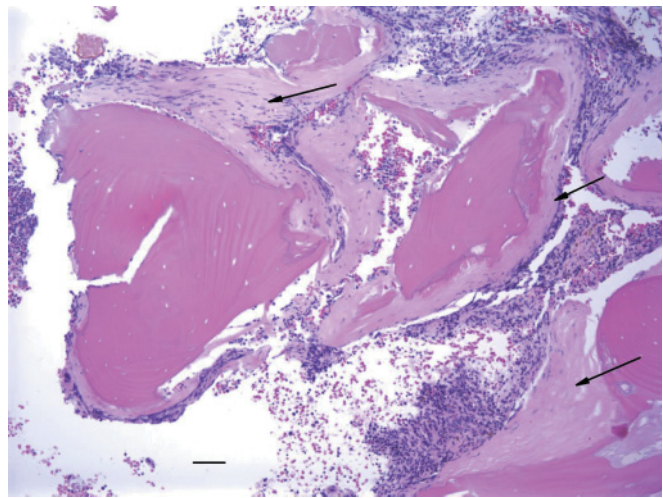


FIGURE 18.4 Paratrabeular fibrosis (arrows) in a bone marrow core biopsy section from a dog. Hematoxylin & Eosin stain; bar, 200 μ m.

to be a reactive change frequently resulting from acute bone marrow injury.^{32,34,38} As such, the fibrosis is regarded as a stage of the repair process much as occurs in other tissues. If the noxious agent is removed or effectively treated, the fibrosis is frequently reversible with the tissue reverting to normal hematopoietic tissue.^{28,32}

Secondary myelofibrosis has been identified in several species including dog, cat, goat, and horse.^{1,34,38} Multiple causes of secondary myelofibrosis have been identified. Fibrosis is frequently present in the subacute or chronic stages of myelonecrosis.^{34,38} The necrosis may or may not be evidenced when the fibrosis is identified but if present is evidence of ongoing bone marrow injury.

In dogs, secondary myelofibrosis has been associated with IMHA, acute leukemias/lymphomas, idiopathic

myelonecrosis, congenital pyruvate kinase deficiency, congenital non-spherocytic anemia, non-hemic malignant tumors, whole body irradiation, and certain drug treatments.^{20,27,32,38,41} Drugs associated with myelofibrosis include phenobarbital, phenylbutazone, and cholchicine (see Chapter 16).³² Approximately half the cases of secondary myelofibrosis are associated with IMHA and intramedullary and extramedullary neoplasia.³² Most affected dogs have a moderate to severe non-regenerative anemia, some are thrombocytopenic, but leukopenia is a rare finding. Ovalocytes are infrequently seen in the peripheral blood. In one study of 19 dogs with secondary myelofibrosis, half of the dogs recovered from their anemia with supportive therapy. When the tumor-associated group was eliminated, 66% of dogs survived more than 8 months. Treatments with immunosuppressive doses of prednisone or with erythropoietin may be useful.

A periparturient myelofibrosis, associated severe non-regenerative anemia, has been reported in young female beagle dogs.²¹ Myelonecrosis was identified in 1 of 3 dogs reported; therefore the fibrosis may have occurred secondary to acute bone marrow injury.

Secondary myelofibrosis is a frequent finding in cat bone marrow.^{3,38} Both collagen and reticulin fibrosis are frequent causes of failure to obtain a sample when aspirating cat bone marrow. Major causes of secondary myelofibrosis in cats include myelodysplastic syndromes, acute myeloid leukemia, IMHA, feline infectious peritonitis, and chronic renal failure.^{3,32} Myelonecrosis is present in many cats with secondary myelofibrosis suggesting that the fibrosis occurs in response to necrosis.

Myelofibrosis has been reported as an inherited disorder in pigmy goats.⁵ At birth, these goats have a non-regenerative anemia, neutropenia, and thrombocytopenia. All animals die at 6–12 weeks of age. Severe myelofibrosis was identified at necropsy. Other necropsy findings included extramedullary hematopoiesis and megakaryocytic hyperplasia and dysplasia.

Myelofibrosis has been described in aged mice.²⁵ Myelofibrosis was observed in 43% of female mice but <1% of male mice. In another study of aged NMRI mice, >90% of females and ovariectomized females had evidence of myelofibrosis as well as some castrated males.²⁴ This study indicates that estrogen may not be a major factor in development of myelofibrosis.

The etiopathogenesis of secondary myelofibrosis is uncertain. The coexistence of myelofibrosis and myelonecrosis in many dog and cat bone marrow specimens indicates that fibrosis may be equivalent to scar tissue in other damaged tissues. Several fibrogenic cytokines have been identified. These include transforming growth factor- β , platelet-derived growth factor, and epidermal growth factor. In bone marrow, fibrogenic cytokines are produced by megakaryocytes and macrophages. Excessive production of fibrogenic cytokines by megakaryocytes is thought to induce myelofibrosis in human idiopathic myelofibrosis, acute megakaryoblastic leukemia, and subtypes of myelodysplastic

syndrome characterized by dysmegakaryopoiesis.²³ Constant high levels of thrombopoietin in mice also induce megakaryocyte hyperplasia and myelofibrosis.^{6,44} Myelofibrosis has been induced in rodents by injection of a variety of cytokines, including thrombopoietin, erythropoietin, and megakaryocyte growth factor.^{44,45} Increased megakaryocytes or dysmegakaryopoiesis has been seen infrequently in dogs with secondary myelofibrosis.³² Hemosiderosis has also been incriminated as a cause of secondary myelofibrosis. Increased hemosiderin has been identified in 28% of dogs with secondary myelofibrosis.

OSTEOSCLEROSIS

Osteosclerosis (also termed myelosclerosis) is a condition of excessive and unorganized production of trabecular bone. Alternatively osteopetrosis is a congenital condition of dogs and humans characterized by severe non-regenerative anemia or pancytopenia and generalized increased bone density.¹⁷ Osteosclerosis appears as irregular fingers or spurs of bone extending from the trabeculae into the hematopoietic space (Fig. 18.5). These sites usually contain increased numbers of osteoblasts and osteoclasts.²⁰ When severe, the associated marked thickening of bone can be visualized radiographically.^{9,15,20} Osteosclerosis is almost always associated with concurrent severe myelofibrosis. Severe osteosclerosis can obliterate or markedly reduced the size of the hematopoietic space. Osteosclerosis along with severe myelofibrosis occurs terminally in dogs with pyruvate kinase deficiency and has been associated with feline leukemia virus (FeLV) infection.^{9,15,20} These changes are seen in core biopsy sections as markedly thickened trabeculae with irregular edges that contain increased numbers of osteoblasts.

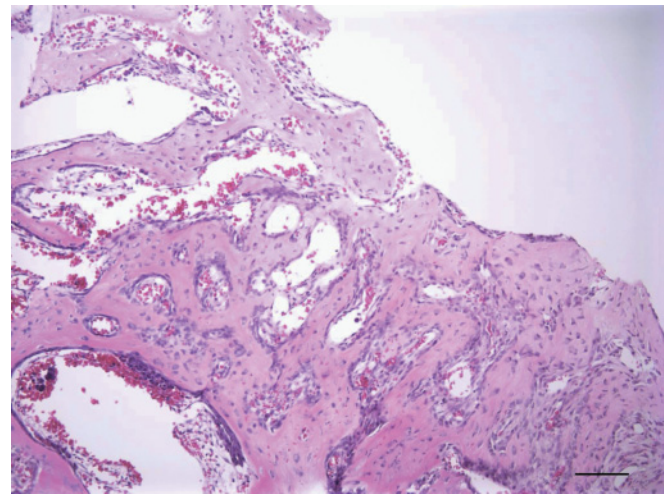


FIGURE 18.5 Osteosclerosis in a bone marrow core biopsy section from a feline leukemia virus-infected cat. Hematoxylin & Eosin stain; bar, 200 μ m.

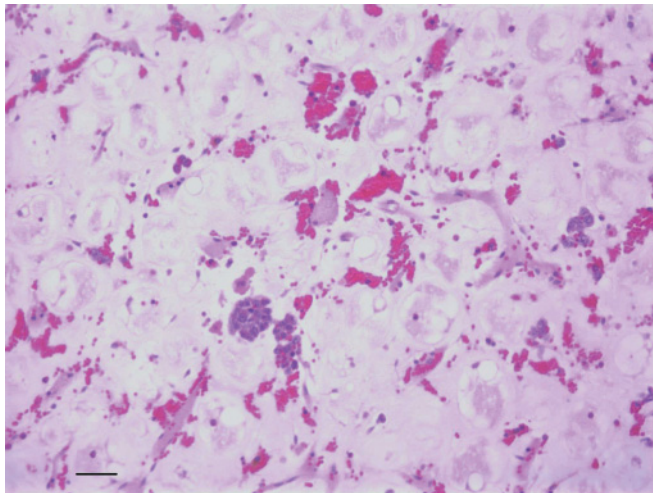


FIGURE 18.6 Gelatinous transformation in a bone marrow core biopsy section from a cat with chronic renal failure. Hematoxylin & Eosin stain; bar, 100µm.

SEROUS ATROPHY OF FAT/GELATINOUS TRANSFORMATION

The paratrabecular space within normal marrow is occupied almost totally by hematopoietic cells or adipocytes. In processing paraffin-embedded tissues the lipid is dissolved out. Therefore, the space within adipocytes normally appears clear and homogeneous. Serous atrophy of fat/gelatinous transformation is a condition in which there is loss of fat cells and hematopoietic cells from bone marrow and replacement by increased amounts of ground substance (Fig. 18.6).²⁶ This is usually associated with starvation or cachexia. Foci of gray or pink gelatinous material may be grossly visible in bone marrow at necropsy. Histopathologic sections of bone marrow are characterized by marrow hypoplasia/aplasia, fat atrophy, and amorphous granular extracellular material. This material is thought to be composed of acid mucopolysaccharides.²⁶

Serous atrophy of fat/gelatinous transformation has been documented in sheep, goats, primates, and cats.^{29,40} In sheep and goats, it has been associated with neglect or loss of teeth. In cats, it has been associated with cachexia associated with the combination of chronic disease and prolonged anorexia. Affected cats had pancytopenia in the blood and marked hypoplasia of bone marrow. Associated conditions included chronic renal failure and oral and gastric ulcers.

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Infectious Injury to Bone Marrow

K. JANE WARDROP

See Chapters 17 and 18
 Rickettsial Infection
 Protozoal Infection – Leishmania
 Viral Infection
 Parvovirus

Retrovirus
 Equine Infectious Anemia virus
 Bovine Viral Diarrhea Virus and Classical Swine
 Fever Virus
 Fungal Infection

Acronyms and Abbreviations

BLV, bovine leukemia virus; BMSC, bone marrow stromal cell; BVDV, bovine viral diarrhea virus; CFU-GM, colony-forming units-granulocyte-monocyte; CPV, canine parvovirus; CSFV, classical swine fever virus; EIAV, equine infectious anemia virus; FeLV, feline leukemia virus; FIV, feline immunodeficiency virus; FPV, feline panleukopenia virus; PCR, polymerase chain reaction.

The response of the bone marrow to infectious agents is variable, depending on the organism involved, the nature and chronicity of infection, and the presence of other diseases. Responses are generally nonspecific, and can occur with other, non-infectious disease. Visualization of the organism on marrow cytology or histology can occur in some infections, facilitating the diagnosis. The marrow response to common companion or domestic animal infectious diseases known for their marrow involvement is described below.

RICKETTSIAL INFECTION

Bacteria within the families *Rickettsiaceae* and *Anaplasmataceae* of the order *Rickettsiales* cause a variety of diseases in dogs, cats, horses, and cattle; however, marrow changes are best documented for infections caused by *Ehrlichia canis*. *Ehrlichia canis* is the etiologic agent of canine monocytic ehrlichiosis, and is transmitted most commonly by the vector *Rhipicephalus sanguineus*. The disease has acute, chronic, and subclinical syndromes. Bone marrow in the acute phase of the disease is typically hypercellular. An increase in the bone marrow myeloid to erythroid ratio was described 3–4 weeks post-infection in experimental canine ehrlichiosis.¹⁷ Megakaryocytes were increased 1 week after infection, reflecting the hematopoietic response to the persistent thrombocytopenia seen with this disease (see Chapter 78). Morulae can be present in bone marrow

but are difficult to find, with 1–4 morulae per 1,000 oil immersion fields seen in one study of acute ehrlichiosis.¹¹ Chronic ehrlichiosis produces a pancytopenia, secondary to severe bone marrow hypocellularity (see Chapter 31). Marrow aspirates are poorly cellular, with cells consisting of low numbers of stromal cells, fibroblasts, and macrophages. Hematopoietic precursors are few or absent. Core biopsies show a severely hypocellular marrow filled with adipose tissue.³ Mastocytosis also has been reported in the chronic phase of ehrlichiosis.¹³ Bone marrow plasmacytosis has been reported with ehrlichiosis, and is independent of the phase.^{12,25}

PROTOZOAL INFECTION – LEISHMANIA

Leishmaniasis is a disease caused by protozoal organisms of the genus *Leishmania*, and is transmitted in Mediterranean regions by the bite of an infected female sand fly. The vector in North America is not known. Several forms of the disease, including cutaneous, mucocutaneous, and visceral forms, have been identified in dogs. A mild anemia and thrombocytopenia are the most frequent hematologic findings. Microscopic examination of fine needle aspirates of lymph nodes or bone marrow, along with serology and polymerase chain reaction (PCR), are frequently used diagnostic methods (Fig. 19.1). In one study, *Leishmania* spp. amastigotes were observed in the marrow of 92.6% of clinically affected dogs.²⁰ Severe histiocytosis of the marrow has been reported.¹⁴

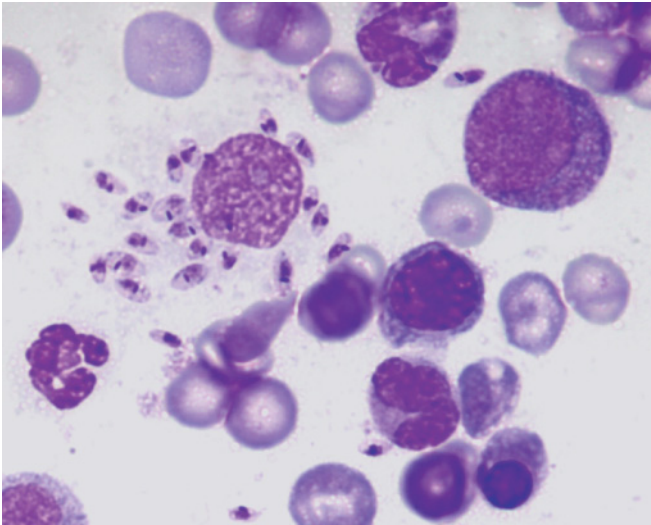


FIGURE 19.1 *Leishmania* organisms in bone marrow from a dog; Wright-Giemsa stain. (Courtesy of Dr. Rick Cowell, Idexx Laboratories.)

VIRAL INFECTION

Parvovirus

Feline panleukopenia virus (FPV) and canine parvovirus (CPV) are both parvoviruses that cause bone marrow injury and leukopenia, neutropenia, and lymphopenia. These viruses have a broad tropism for mitotically active cells. It is unknown whether the reduction in marrow cells is due primarily to virus replicating in and killing cells, or due to more indirect effects. FPV can kill both erythroid and myeloid colony progenitor cells in bone marrow cultures, although at low viral doses there is greater suppression of the formation of myeloid colony-forming units-granulocyte-monocyte (CFU-GM).⁷ Viral replication of FPV in myeloid cells may lead to the neutropenia seen after FPV infection. In a study of 76 cats with clinical FPV, 84% showed complete bone marrow depopulation and dilation of the vascular sinuses, 5% had no significant marrow change and 6% had marrow hypercellularity with granulocytic hyperplasia.⁴

In 75 dogs with clinical CPV, 92% had marrow depopulation, one dog showed no changes, and five dogs had marrow hypoplasia.⁴ In another study using experimentally infected puppies, leukopenia and neutropenia only occurred in dogs with severe enteric disease. Viral replication in the bone marrow was sparse.⁹ In both FPV and CPV, the severe neutropenia observed is probably the result of both neutrophil loss or consumption in the infected gut and bone marrow suppression.¹⁵

Retrovirus

Feline leukemia virus (FeLV, subfamily *Oncornavirinae*) and feline immunodeficiency virus (FIV, subfamily *Lentivirinae*) are retroviruses that are major infectious

pathogens in domestic cats (see Chapters 55 and 62). FeLV infects hematopoietic cells, lymphoid cells, and accessory cells in the hematopoietic microenvironment. Marrow nucleated cells become infected with the virus 2–6 weeks after exposure, and virus can be detected in circulating leukocytes at that time.⁸ After regression, one third to one half of cats transiently harbor latent virus in marrow myelomonocytic cells and stromal fibroblasts.⁸ The types of marrow change seen in FeLV are highly variable. Early viremia can be accompanied by pancytopenia and marrow hypocellularity.¹⁶ Marrow necrosis, with nuclear swelling and karyolysis has rarely been described.²² FeLV-C-induced pure red cell aplasia shows almost a total lack of marrow erythroid precursor cells, with normal myeloid and megakaryocytic precursors (see Chapters 38 and 62).²¹ FeLV-induced myelodysplastic syndromes can show marrow hypercellularity, megaloblastic maturation abnormalities, increased reticulin fibrosis, and increased numbers of immature cells and blast cells (see Chapter 66).^{2,10} Marrow myelophthitic disease, where the marrow is filled with blast cells, can be observed in FeLV-induced leukemias or lymphoma.

FIV produces progressive CD4 T cell lymphopenia. Marrow morphologic abnormalities, marrow suppression, and peripheral blood cytopenias can be seen. Cytopenias (neutropenia, anemia) have been attributable to direct infection of the bone marrow stromal cell (BMSC) population.¹⁹ BMSC types include fibroblastic/advential/reticular cells, macrophages, endothelial cells, adipocytes, and myofibroblasts, of which macrophages are productively infected with FIV.¹

Bovine leukemia virus (BLV) is an oncogenic retrovirus which produces lymphoma in adult cattle. Bone marrow involvement is not a prominent feature.

Equine Infectious Anemia Virus

The equine infectious anemia virus (EIAV) is a lentivirus of the family *Retroviridae* (see Chapters 32 and 35). Infection of horses with EIAV produces a persistent viremia and recurrent episodes of anemia, thrombocytopenia, and fever. In one study using experimentally infected foals with severe combined immunodeficiency, bone marrow changes were described after the onset of thrombocytopenia.²⁴ The post-thrombocytopenia samples had a moderate to marked decrease in bone marrow cellularity, with an increase in intercellular homogeneous eosinophilic material. Individual cell necrosis or apoptosis was not observed. A moderate increase in adipocytes and reticulin fibers was noted. Denuded megakaryocyte nuclei were also observed. In another study, the total megakaryocyte area and megakaryocyte nuclear area were increased. Numbers of megakaryocytes were slightly increased.¹⁸

Bovine Viral Diarrhea Virus and Classical Swine Fever Virus

Bovine viral diarrhea virus (BVDV) and classical swine fever virus (CSFV) are related viruses (family

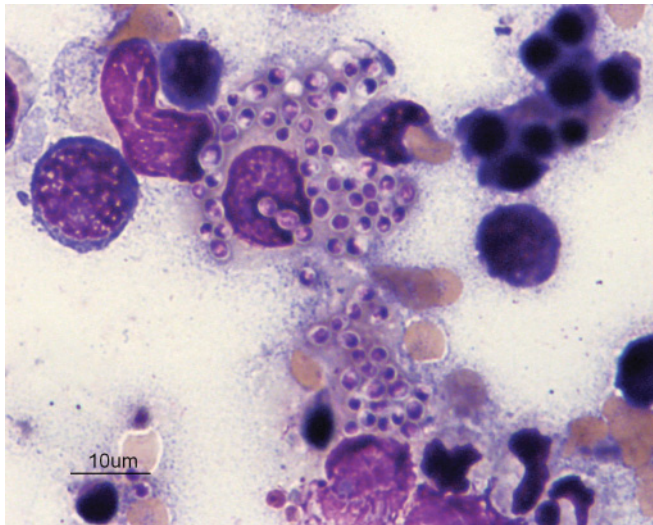


FIGURE 19.2 Histoplasma organisms in bone marrow from a cat; Wright-Giemsa stain. (Courtesy of Dr. James Meinkoth, Oklahoma State University.)

Flaviviridae, genus Pestivirus) both known to infect and damage megakaryocytes of the marrow and produce a subsequent thrombocytopenia, but other marrow lesions are not present. In a study using calves infected with type II BVDV, histologic evaluation of the bone marrow showed an increased number of megakaryocytes. Approximately one-half of the megakaryocytes were immature or had undergone nuclear loss or pyknosis.²³ Infection of calves with type II BVDV also reduced the number of GFU-GM cultured from bone marrow. This suggests that suppression of granulopoiesis may contribute to the prolonged neutropenia associated with BVD infection. Experimental infection of pigs with a virulent strain of CSFV also produced changes in megakaryocytes, with abnormal nuclei and micromegakaryocytes seen.⁶

FUNGAL INFECTION

Blastomycosis, histoplasmosis, coccidiomycosis, and cryptococcosis are common systemic fungal diseases of the dog and cat. Although all of these diseases can involve the bone marrow, marrow involvement is most frequently described in histoplasmosis.

Histoplasma capsulatum is a soil-borne, dimorphic fungus that infects dogs and cats through inhalation of conidia from the mycelial phase, which subsequently convert to yeast in the body. The disseminated form of the disease predominantly affects the liver, spleen, gastrointestinal tract, bone, bone marrow, integument, and eyes. Primary gastrointestinal and pulmonary histoplasmosis can also occur.

In the bone marrow, large numbers of organisms can be found within macrophages (Fig. 19.2). Anemia is a frequent finding, but pancytopenia accompanied by granulomatous inflammation of the bone marrow has also been reported.⁵

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A microscopic image of a blood smear. The background is filled with numerous red blood cells (erythrocytes) of varying sizes and shapes. Several white blood cells (leukocytes) are visible, including a large cell with a multi-lobed nucleus (neutrophil) in the upper left, a cell with a large, dark, kidney-shaped nucleus (monocyte) in the lower left, and a cell with a large, dark, round nucleus (lymphocyte) in the lower right. The text is overlaid on the right side of the image.

SECTION III

Erythrocytes
Joanne B. Messick

Erythrocyte Structure and Function

CHRISTINE S. OLVER, GORDON A. ANDREWS, JOSEPH E. SMITH, and J. JERRY KANEKO

Erythrocyte Structure

Overall Structure

Membrane Structure

Membrane lipids

Lipid rafts

Maintenance of membrane fluidity

Cytoskeletal proteins

Integral membrane proteins

Hemoglobin Structure, Synthesis, and Metabolism

Hemoglobin Structure

Hemoglobin Synthesis

Control of Hemoglobin Synthesis

Hemoglobin Turnover

Heme Catabolism and Bilirubin Formation

Fetal Hemoglobin

Hemoglobin Types in Animals

Iron Metabolism

Absorption in Intestine

Intestinal absorption of non-heme iron

Cellular Iron Uptake

Molecules Involved in Iron Metabolism

Divalent metal iron transporter-1

Ferrireductase duodenal cytochrome b

Hephaestin

Ferroportin

Hepcidin

Transferrin receptor

Transport and Storage of Iron

Transport of iron

Storage of iron

Genetic Control of Iron Regulatory Proteins

Acronyms and Abbreviations

dCytb, duodenal cytochrome B; DMT-1, divalent metal iron transporter-1; Hgb, hemoglobin; ID, iron deficiency; IRP, iron regulatory protein; kD, kilodalton; MPS, mononuclear phagocyte system; NADH, nicotinamide adenine dinucleotide, reduced form; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; RBC, red blood cell; SM, sphingomyelin; TfR, transferrin receptor.

The main function of red blood cells (RBCs) is to carry oxygen to tissues. All energy in RBCs that is devoted to maintaining cell shape, membrane structure, enzymatic functions, reduced iron in hemoglobin and other functions does so to optimize oxygen delivery to tissues.

ERYTHROCYTE STRUCTURE

Overall Structure

RBCs have no nuclei and no organelles, and thereby no ability to synthesize proteins. The full complement of functional proteins must be present by the time the reticulocyte matures. RBCs are composed (by mass)

of 61% water, 32% protein (mostly hemoglobin), 7% carbohydrates, and 0.4% lipids. Isolated RBC membranes in most animals are composed of approximately 20% water, 40% protein, 35% lipid and 6% carbohydrate.⁴³

Membrane Structure

Membrane Lipids

The RBC membrane phospholipid bilayer is composed of phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), and phosphatidylserine (PS), as well as other less abundant phospholipids.⁵⁹ This composition varies with species. For instance, sheep have no PC and camellids have very low PC,

whereas rats have more PC.^{39,51,59} The phospholipids have polar head groups and hydrophobic tail groups. The tail groups associate together in the bilayer to provide a hydrophobic barrier to water while the polar groups face the hydrophilic exterior. The bilayer is vertically asymmetrical, with PS and PE located almost exclusively on an inner PL layer. This asymmetry is maintained by the activities of enzymes termed flippase¹⁴ and floppase.^{33,50} There is also horizontal asymmetry, so that there is lipid composition variability within different domains of the bilayer. Other lipids in the bilayer include glycolipids and cholesterol, and these are embedded in the phospholipids.

Lipid Rafts

Lipid rafts are specialized membrane microdomains that are rich in cholesterol and sphingolipids and also contain specific membrane proteins. They are isolated as detergent resistant membranes because of their insolubility in non-ionic detergents.²⁹ Examples of proteins localized in these microdomains include glycosphosphatidylinositol-anchored proteins, flotillins, stomatin, and aquaporin-1.⁴⁵

Maintenance of Membrane Fluidity

Anything that increases the “packing” of the membrane components together will “harden” the membrane, and anything that decreases the packing will “soften” the membrane. Membrane fluidity is, therefore based on several factors. One factor is the degree to which fatty acid chains are esterified with cholesterol. Esterified cholesterol is bulkier, and therefore “packs” more loosely, and creates a more fluid membrane. A second factor is the class of phospholipid and the type of fatty acid contained within the membrane. A third factor is the molar ratio of cholesterol to phospholipids. A fourth factor is the degree of saturation of phospholipid fatty acids (saturation makes PLs “pack” better and, therefore decreases fluidity).

Cytoskeletal Proteins

Cytoskeletal proteins are located on the cytoplasmic side of the plasma membrane and function to help maintain cell shape, membrane deformability, membrane stability, and the lateral mobility of some integral membrane proteins.⁶² They are called “peripheral proteins” because they are not embedded within the membrane but rather are anchored to the cytoplasmic side of the bilayer.

Alpha and beta spectrin subunits are the largest and most abundant proteins in the cytoskeleton. They are elongated and self-associate to form $\alpha_2\beta_2$ tetramers which then associate end to end into a hexagonal network of molecules that lies just underneath the plasma membrane. The spectrin lattice is anchored to other proteins and to the bilayer at select areas of the hexagon. The spectrin molecules are secured to the lipid bilayer at “nodes” called junctional complexes (Fig.

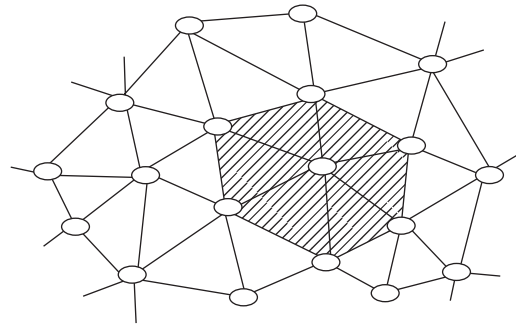


FIGURE 20.1 Lattice structure provided by spectrin tetramers in the cytoskeleton of erythrocytes.

20.1). These complexes are made up of actin, 4.1 protein (named for its migration during one dimensional electrophoresis), and the integral membrane protein glycoporphin. Spectrin is also affixed to the inner leaflet via an interaction with ankyrin (a peripheral protein) that is bound to band 3 (anion transporter, an integral membrane protein). Additional peripheral membrane proteins comprising the cytoskeleton include adducin, tropomyosin, tropomodulin, and palladin.

Integral Membrane Proteins

Band 3 or the anion exchange protein is an integral membrane protein composed of dimers, tetramers and other large oligomers. It is responsible for anchoring the cytoskeleton to the membrane, anion exchange and binding of glycolytic enzymes, hemoglobin and hemichromes. The anion exchange function is primarily for delivering CO_2 to the lungs in exchange for Cl^- .

Glycophorins are heavily glycosylated integral membrane proteins. They carry most of the sialic acid residues and, therefore negative charge on the surface of the RBC. RBCs carry other cell surface molecules, and new ones are being discovered all the time. A select list of these is shown in Table 20.1.

HEMOGLOBIN STRUCTURE, SYNTHESIS, AND METABOLISM

Hemoglobin is an iron-porphyrin-protein complex. Other iron-porphyrin-protein complexes include myoglobin and heme-containing enzymes such as catalase, peroxidase, and cytochromes. The porphyrin-iron-protein hemoglobin molecule occupies a central role in physiology by binding, transporting, and delivering oxygen to tissues. Hemoglobin is synthesized within developing RBCs and its synthesis is coordinated with the developmental stages of the erythroid precursors.

Hemoglobin Structure

Hemoglobin is composed of two α - and two β -polypeptide chains and is approximately 64kDa in molecular weight. Each chain contains a heme prosthetic group held firmly within a hydrophobic cleft

TABLE 20.1 Selected Erythrocyte Surface Molecules in the Human and/or Mouse

Molecule	Synonyms	Function
CD35	Complement receptor 1	Binds complement fragments
CD36	Platelet glycoprotein IV	Unknown
CD38	N/A	NAD glycohydrolase
CD44	H-CAM, gp85	Hyaluronic acid binding
CD47	Integrin associated protein	May be a senescence marker
CD49d/CD29 ^a	VLA-4, $\alpha\beta 1$ integrin	Adhesion to fibronectin, V-CAM
CD55	Decay accelerating factor	Neutralizes complement activation
CD59	Complement regulatory protein	Inhibits complement membrane attack
CD71 ^a	Transferrin receptor	Iron acquisition
CD147	N/A	Recirculation of RBC from spleen
CD239	Basal cell adhesion molecule/Lutheran	Laminin binding

^aReticulocytes only.

(otherwise known as the “heme pocket”). The entire molecule is, therefore a globular tetramer. This globular structure permits a cooperative interaction of oxygen binding that gives the sigmoid oxygen-Hgb saturation curve.

Hemoglobin Synthesis

Heme is a planar molecule composed of two elements, a tetrapyrrole protoporphyrin IX and a central iron molecule. The iron is supplied by ferritin, the cytosolic storage form of iron. Heme biosynthesis is an enzymatic process requiring both mitochondrial and cytosolic enzymes. Figure 20.2 shows the sequence of reactions leading to the synthesis of PROTO IX, heme, and Hgb. The initial step of heme synthesis occurs in mitochondria. It requires vitamin B₆ (pyridoxine) and is catalyzed by δ -aminolevulinic acid (ALA) synthase. ALA is then translocated to the cytosol. In the cytosol, two moles of ALA are condensed to form porphobilinogen (PBG), a reaction catalyzed by ALA-dehydrase (ALA-D). ALA-D is strongly inhibited by lead, leading to the anemia associated with lead poisoning. The remainder of the process is depicted in Figure 20.2.

Synthesis of α and β globin chains occurs in the ribosomes and polyribosomes in the cytoplasm. Each globin chain contains a cleft or heme pocket that is lined by largely nonpolar amino acid side chains that impart a hydrophobic nature to the pocket. The nonpolar (vinyl and methyl groups) side of heme is positioned in the hydrophobic pocket of each globin chain. The addition of heme to the pockets of globin is followed by a dimerization of the α and β globin chains, which is then followed by spontaneous formation of hemoglobin tetramers.

Control of Hemoglobin Synthesis

Synthesis of heme and globin chains is finely coordinated so that there is little or no free heme or globin in the cytoplasm of developing erythroid cells. Heme

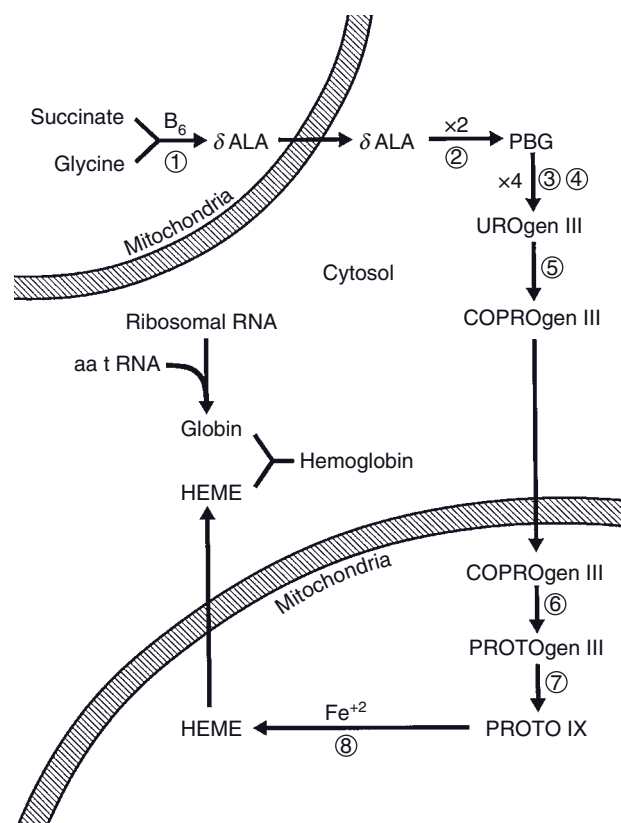


FIGURE 20.2 Pathway for protoporphyrin, heme, and hemoglobin synthesis emphasizing the partitioning of the pathway between the mitochondrial and the cytosolic compartments of the cell. The circled numbers represent the following enzymes of heme synthesis: 1, ALA-Syn; 2, ALA-D; 3, UROgenI-Syn; 4, UROgenIII-Cosyn; 5, UROgen-D; 6, COPROgenIII-Ox; 7, PROTOgenIII-Ox; 8, FER-Ch. (Courtesy of Christine S. Olver)

governs ribosomal translation of globin chain synthesis.⁵⁷ Moreover, α and β chain synthesis is coordinated. Excess α chains inhibit their own synthesis while stimulating β -chain synthesis, while excess β chains inhibit their own synthesis.^{27,54}

Hemoglobin Turnover

Hemoglobin is turned over after the extra- or intravascular destruction of RBCs. In health, extravascular hemolysis occurs as RBCs age and are subsequently trapped in the spleen and phagocytosed by splenic macrophages. Hemoglobin is catabolyzed within a cell. When RBCs undergo intravascular hemolysis, the hemoglobin is released into the circulation in its free form. Free plasma Hgb is quickly cleared by several mechanisms. The most important is binding of free Hgb to haptoglobin followed by clearance of the complex by macrophages.²⁶ The half-time for free Hgb clearance is 20–30 minutes. Minor pathways for Hgb clearance are (1) oxidation to methemoglobin (MetHgb) that can be excreted, (2) hydrolysis of the MetHgb to release its ferriheme (ferric heme, hematin, hemin) that is complexed to hemopexin for transport to the mononuclear phagocyte system (MPS), and (3) binding of ferriheme to albumin (methemalbumin) for transport to the MPS. When there is excess intravascular hemolysis, such that the binding capacity of plasma haptoglobin is exceeded, Hgb may be cleared via glomerular filtration. In this event, hemoglobinuria may occur, or the proximal renal tubular cells may catabolyze Hgb.¹⁶ When Hgb is internalized into macrophages, it is degraded by hydrolysis into its globin and heme moieties. The globin chains are systematically degraded by proteolytic enzymes to release the constituent amino acids.

Heme Catabolism and Bilirubin Formation

The initial step in the heme catabolic pathway is enzymatic cleavage of the heme ring at the α methene bridge to release linear tetrapyrrole biliverdin, iron, and carbon monoxide. This reaction is catalyzed by microsomal heme oxygenase in the presence of cytochrome P-450, oxygen, and reduced NADPH. Heme oxygenase activity is highest in the spleen, and there is some activity in the liver, bone marrow, and renal tubular cells. Iron is oxidized to the ferric form, released and transported as transferrin for storage as hemosiderin or ferritin in the liver and as ferritin in the bone marrow for subsequent reuse.⁵⁵ Biliverdin, a green pigment, is reduced in macrophages to bilirubin, a yellow pigment, by the action of the enzyme biliverdin reductase in the presence of NADPH. The virtual absence of biliverdin reductase in birds accounts for the green color of avian bile. Bilirubin is a nonpolar compound and must be bound to albumin to remain soluble in aqueous plasma. The bilirubin-albumin complex is transported to the liver, and at the hepatocyte surface, albumin is released. The first step involving the hepatocyte surface is the uptake or transport of bilirubin across the hepatocyte membrane by a transporter system. The second step in the hepatocyte is conjugation of bilirubin to glucuronic acid, primarily as the diglucuronide and with some monoglucuronide. This reaction is catalyzed by the enzyme glucuronyl transferase. The bilirubin glucuronides are highly polar and are therefore soluble compounds. The third step in the hepatocyte is the transport

of bilirubin glucuronide into the bile canaliculi and thence the biliary system. This canalicular transport is the rate-limiting step in hepatic bilirubin metabolism.²⁸ A small amount of the glucuronides may also be transported back into the circulation so that normally, both unconjugated and conjugated bilirubins are present in the circulation.

Fetal Hemoglobin

The synthesis of α globin chains remains constant during stages of gestational development and throughout adult life. Different types of non- α globin chains are synthesized and account for the different types of Hgb appearing during development and in adult life. At the embryonic stage, most animals synthesize only embryonic chains designated ϵ chains (i.e. embryonal Hgbs). Fetal Hgb (HgbF) is composed of two γ chains and two α chains, although not all animals produce HgbF. Ruminants possess a mixture of adult and fetal Hgb at birth, and HgbF is replaced by adult Hgb in the first few months of life.³ Although HgbF rapidly declines after birth, the capacity to synthesize it remains, and HgbF may appear in responsive anemias. Dogs, cats, horses, and pigs lack HgbF and are born with adult Hgb that has replaced the embryonal Hgb during gestation.^{9,31}

Hemoglobin Types in Animals

With the possible exception of pigs, two or more types of Hgb normally occur in several domestic animal species.^{8,30} Most polymorphisms are determined genetically and are usually associated with multiple amino acid substitutions.³¹ The Hgb of cats has a unique structure containing 8–10 reactive sulfhydryl groups per molecule compared to other animals that have only 2–4 per Hgb molecule.³⁶ The presence of the readily oxidizable groups is regarded as the basis for the ease of Heinz body formation in the cat (see Chapter 36). Sheep and goats synthesize HgbC in response to severe anemia.²⁵ Sheep normally have HgbA and goats HgbA and HgbB, and the switch to HgbC during anemia is mediated by erythropoietin.⁴ Carbon dioxide decreases oxygen affinity for HgbC more than it does for normal adult Hgbs.^{25,63}

IRON METABOLISM

Iron is an essential component not only of hemoglobin and myoglobin, but also of many enzymes. Iron containing enzymes include NADH dehydrogenase, lipoxygenases, superoxide dismutase, ribonucleotide reductase, fatty acid desaturases and phosphatases.³⁷ These enzymes function in energy generation, prostaglandin synthesis, free radical detoxification, synthesis of DNA, synthesis of fatty acids, and signal transduction. Iron is provided to cells of the body by three sources: (1) absorption from the gut, (2) recycling from senescent RBCs, and (3) liver storage iron (Fig. 20.3).

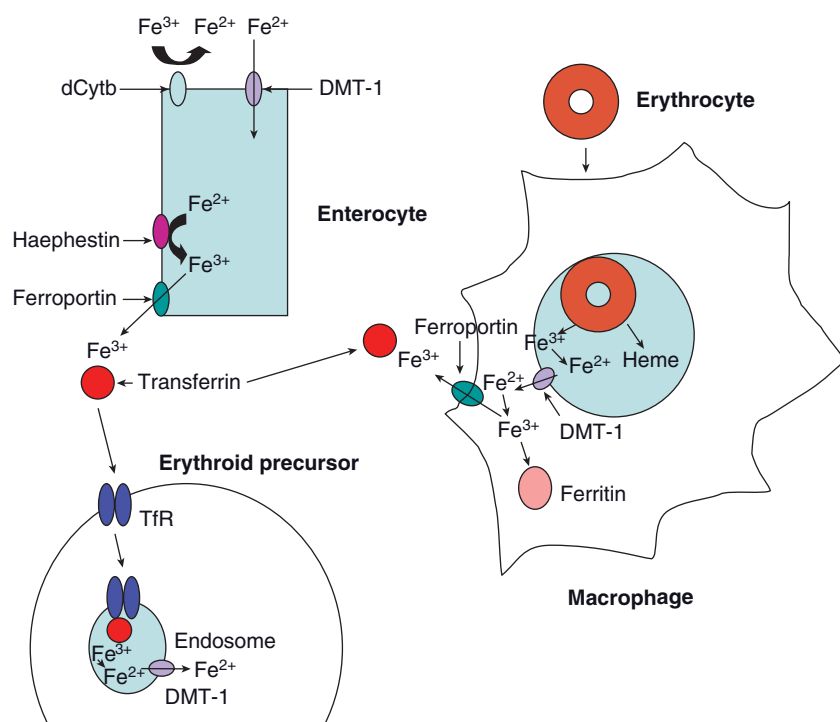


FIGURE 20.3 A schematic of iron metabolism including absorption from enterocytes, transport in circulation, utilization by the erythroid precursors, and recycling by macrophages.

Absorption in Intestine

Absorption of iron is regulated; excretion is not (iron is lost through epithelial sloughing of gut, skin, urothelium). Iron absorption takes place in the proximal duodenum, and it is absorbed either as heme or non-heme iron. Although most iron utilized in physiologic mechanisms is that recycled from hemoglobin iron, total body iron stores are regulated by the duodenum to prevent whole-body iron accumulation or deficit.

Intestinal Absorption of Non Heme Iron

Iron is stabilized in the ferric form by the acidity of the gastric fluid. Apical duodenal cytochrome B (dCytb) reduces ferric to ferrous iron. Ferrous iron is transported to the basolateral membrane by intracellular mechanisms that are unclear. At the basolateral surface, hephaestin, a copper-containing enzyme, re-oxidizes iron to its ferric state. Ferroportin then exports iron out of the cells into the portal circulation, where it is carried bound to transferrin. Once in the circulation, iron is carried to the liver to be stored, or to the erythroid precursors in the bone marrow to be incorporated into hemoglobin. Heme iron is probably absorbed by an apical heme transporter (apical heme carrier protein 1).⁴⁶

Cellular Iron Uptake

Iron is taken up by most cells (i.e. not enterocytes) via the cell surface transferrin receptor (TfR). The expression level of this receptor is directly related to the iron

requirements of the cell.^{6,15} The cell surface transferrin receptor functions as a dimer, with each 90 kDa monomer embedded in the membrane with one single transmembrane-spanning region. Iron binding transferrin (holotransferrin) binds to the TfR at physiologic pH and the transferrin-iron-TfR complex is internalized into an endosome. The low pH of the endosome causes the release of Fe^{3+} from the transferrin and the iron is then reduced to Fe^{2+} . Ferrous iron is then exported to the cytoplasm.^{5,34,41,47} A newly discovered protein, the product of the *HFE* gene, forms a complex with the TfR and appears to regulate its ability to import iron.⁷

Molecules Involved in Iron Metabolism

The uptake of iron by the duodenum and by individual cells in the body is regulated by total body iron levels and intracellular iron levels, respectively.¹

Many molecules involved in iron regulation have been discovered in recent years.^{11,20} Although there are still gaps in our knowledge of the physiologic processes, several important molecules have been identified in research involving humans and rodents, and are described below.

Divalent Metal Iron Transporter-1

Divalent metal iron transporter-1 (DMT-1) is expressed in the enterocytes of the villi and not in the crypts,¹⁰ and is responsible for transporting iron from the enterocyte to the circulation, and from endosomes of macrophages to the cytoplasm. It is associated with phagosomal membranes in macrophages²² and plasma membranes

and endosomes of cells that use the TfR complex for iron acquisition.²² One isoform of DMT-1 is strongly regulated by iron; it is markedly increased with an iron-deficient diet and reduced by an iron replete diet.²³

Ferrireductase Duodenal Cytochrome b

DMT-1 transports Fe^{2+} into the enterocyte from the duodenal lumen. Since Fe^{3+} is the dietary form that exists in the lumen, it must be reduced. The duodenal cytochrome b, located on the apical membrane of the enterocyte, acts as such a reductase.¹⁸

Hephaestin

Hephaestin, a copper dependent transmembrane ferroxidase, is an approximately 150–160 kDa protein with 50% homology to ceruloplasmin. It is highly expressed in the small intestine, primarily in the basolateral membrane of enterocytes, and is co-localized with ferroportin.⁴⁴ Hephaestin is responsible for oxidizing Fe^{2+} to Fe^{3+} so that it can be transported out of the enterocyte into the plasma.

Ferroportin

Ferroportin is expressed on the membrane of duodenal enterocytes (basolateral), placental trophoblasts, and macrophages distributed in various tissues.^{1,13,17,18} It is the only mammalian iron exporter out of cells. It exports iron from enterocytes into the circulation (from dietary iron) and from macrophages to the circulation (from recycled erythrocyte iron). Its expression levels are regulated by hepcidin binding and internalization of the protein, and also by iron levels.

Hepcidin

Hepcidin should be considered a molecule that regulates iron systemically. Hepcidin regulates iron export from enterocytes and macrophages by binding to and causing degradation of ferroportin.²¹ It is synthesized as a pro-peptide in the liver and circulates as a 25 amino acid disulfide-rich peptide. Synthesis is increased by iron loading to decrease iron absorption, and decreased by anemia and hypoxia to increase iron absorption.^{42,60} It is also increased in inflammation, and causes the iron trapping in macrophages during anemia of inflammatory disease.²¹ Iron may control hepcidin synthesis through a signaling pathway dependent on proteins called hemojuvelin and bone morphogenic proteins (2 and 4).³⁵ Kupffer cells also appear to regulate hepcidin expression; when Kupffer cells are eliminated, hepcidin expression is increased and serum iron is decreased.⁵⁶

Anemia and hypoxia decrease hepcidin synthesis by increasing erythropoietic activity. Erythropoietin administration down-regulates hepcidin expression and this is probably due to the erythropoietic activity and not the direct effects of the hormone.^{32,60}

Inflammation, primarily through interleukin-1 and -6, and possibly transforming growth factor-beta, increases hepcidin expression, thus decreasing intestinal iron absorption and decreasing iron export from macrophages.

Transferrin Receptor

The transferrin receptor is described under the heading Cellular iron uptake.

Transport and Storage of Iron

Transport of Iron

Transport of iron from one compartment or tissue to another through the circulation (e.g. enterocytes to bone marrow cells) is accomplished by the carrier protein transferrin. Serum transferrin is a soluble 78 kDa protein that binds one or two iron atoms in their ferric (3^+) form.

Storage of Iron

Iron is stored in developing RBCs, and other cells, as ferritin, an iron-protein complex.^{38,48,49} The protein moiety, apoferritin, consists of at least 24 monomers of either H or L subunits. Each subunit is shaped like a short rod and these rods are assembled as a hollow sphere. Apoferritin has a molecular weight of 441 kDa and can store as much as 4500 molecules (31%) of iron.³⁴ The maximal weight of ferritin can be approximately 800 kDa, but ferritin more commonly is less than that (620 kDa) with roughly 18% iron. The method of iron movement in and out of the molecule is not precisely known, but it apparently enters as the ferrous form and is oxidized to and stored as the ferric form.¹² Iron is released either by a reversal of the process, or by digestion of ferritin by lysosomes and reduction to the ferrous form.²⁴ Hemosiderin is found in cells of the monocyte-macrophage system, predominantly in macrophages of the liver (Kupffer cells), spleen, and bone marrow. Hemosiderin can be seen as golden granules in hematoxylin and eosin stained histologic sections, and may appear green to black in Wright-Giemsa stained cytologic preparations. It is a “stripped down” version of ferritin, whereby the apoferritin protein shell has been removed, leaving approximately 25–30% of iron by weight.¹⁹

Genetic Control of Iron Regulatory Proteins

Proteins involved in iron metabolism are predominantly regulated at the post-translational level by the interplay of iron-responsive elements (IREs) and cytosolic iron-binding proteins referred to as iron regulatory proteins (IRPs). The IREs are stem-loop or hairpin structures located in the 3' or 5' untranslated regions of the messenger RNAs (mRNAs) that act as nucleic acid binding sites for the IRPs. The binding affinity of IRPs is reversibly regulated by the intracellular

concentration of iron. When the intracellular concentration of iron is low, IRPs have a high affinity for IREs, and when cells are iron replete, the affinity is low.

Ferritin and aminolevulinic synthase (rate limiting enzyme in heme biosynthesis) mRNAs have a single IRE in the 5' end at the beginning of the coding region. Binding of the IRP inhibits translation; thus when iron is low, the IRP binds to the IRE and translation is inhibited, whereas when iron is high, IRPs lose affinity and translation of those proteins occurs.^{2,52,53,58,61} Thus, when ferritin is required for storage of increased intracellular iron, translation is up-regulated. On the other hand, the transferrin receptor mRNA has an IRE on the 3' end. When iron is low, the binding of the IRP inhibits the breakdown of the mRNA and thereby stimulates the translation of the protein. Thus, low iron up-regulates TfR so that cells become more iron avid. In summary, IRPs regulate mRNA translation by binding to IREs, and binding is stimulated by low iron. However, sometimes binding stimulates translation (TfR) and sometimes binding inhibits translation (ferritin).⁴⁰

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Erythrocyte Biochemistry

JOHN W. HARVEY

Membrane Transport
Carbohydrate Metabolism
Embden-Meyerhof Pathway and ATP Production
Diphosphoglycerate Pathway and Oxygen Affinity of Hemoglobin

Oxidants and Oxidative Injury
Pentose Phosphate Pathway and Protection Against Oxidant
Methemoglobin Formation and Reduction

Acronyms and Abbreviations

Cb₅R, cytochrome-b₅-reductase; DPGM, diphosphoglycerate mutase; DPGP, diphosphoglycerate phosphatase activity; 1,3DPG, 1,3-diphosphoglycerate; 2,3DPG, 2,3-diphosphoglycerate; EMP, Embden-Meyerhof pathway; FAD, flavin adenine dinucleotide; GAPD, glyceraldehyde phosphate dehydrogenase; G6P, glucose 6-phosphate; G6PD, glucose-6-phosphate dehydrogenase; GPx, glutathione peroxidase; GR, glutathione reduction; GSH, reduced glutathione; GSSG, oxidized glutathione; HK, hexokinase; HK⁺, high potassium; LK⁺, low potassium; LMB, leu-komethylene blue; MB, methylene blue; O₂⁻, superoxide; PFK, phosphofructokinase; 3PG, 3-phosphoglycerate; P_i, inorganic phosphate; PGK, phosphoglycerate kinase; PK, pyruvate kinase; PPP, pentose phosphate pathway; RBC, red blood cell; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase.

Erythrocytes or red blood cells (RBCs) provide vital functions of oxygen transport, carbon dioxide transport, and buffering of hydrogen ions. These functions do not require energy per se, but energy in the form of ATP, NADH, and NADPH is needed to keep the cells circulating for months in a functional state despite repeated exposures to mechanical and metabolic insults.

Mature mammalian RBCs do not have nuclei; consequently, they cannot synthesize nucleic acids or proteins. The loss of mitochondria during the maturation of reticulocytes results in a loss of Krebs' cycle and oxidative phosphorylation capabilities, and prevents the synthesis of heme or lipids *de novo* in RBCs. Energy needs of mature RBCs are met solely by anaerobic glycolysis in the Embden-Meyerhof pathway (EMP). Although metabolic demands are lower than in other blood cell types, RBCs still require energy in the form of ATP for maintenance of shape, deformability, phosphorylation of membrane phospholipids and proteins, active membrane transport of various molecules, partial synthesis of purine and pyrimidine nucleotides, and synthesis of glutathione.⁹ A minor shunt for carbohydrate metabolism, the pentose phosphate pathway, pro-

vides the RBC with additional critical protection against oxidative damage.

MEMBRANE TRANSPORT

The RBC lipid bilayer is impermeable to most molecules. Consequently, various membrane protein transport systems are utilized for movement of molecules into and out of RBCs. Water and carbon dioxide are transported across RBC membranes using water channels called aquaporins.⁴ Band 3 appears to function as a channel for the movement of anions, especially bicarbonate and chloride, certain non-electrolytes, and probably cations to some extent. Defective anion transport and marked spherocytosis with membrane instability occurs in anemic cattle with an inherited deficiency of band 3 protein (see Chapter 29).¹¹

Major interspecies, and in some cases intraspecies, differences occur in cation transport and subsequently in intracellular Na⁺ and K⁺ concentrations.³ Animal species with high intracellular K⁺ concentrations (horse, pig, and some ruminants) have an active Na⁺,K⁺-pump that exchanges intracellular Na⁺ for extracellular K⁺

with the hydrolysis of ATP. In addition to individuals with high potassium (HK^+) RBCs, some sheep, goats, buffalo, and most cattle RBCs have relatively low potassium (LK^+) and, consequently, high sodium RBCs. These LK^+ RBCs have low Na^+, K^+ -pump activity and high passive K^+ permeability.

Red blood cells from cats and most dogs do not have Na^+, K^+ -pump activity and have Na^+ and K^+ concentrations near those predicted for the Donnan equilibrium with plasma. However, many clinically normal Japanese and Korean dogs have HK^+ RBCs.⁵ Red blood cells from these dogs have substantial Na^+, K^+ -ATPase activity, and some of these dogs also have increased glutamate transport, which results in high reduced glutathione (GSH) concentrations. These HK^+ , high GSH RBCs promote *Babesia gibsoni* replication compared to LK^+ , normal GSH RBCs.²⁴ Other pathways of sodium and potassium transport occur to variable degrees in certain species.⁹

Excessive intracellular Ca^{2+} promotes the suicidal death of RBCs (eryptosis);¹³ consequently, RBCs actively extrude Ca^{2+} using a calcium pump having Ca^{2+} -activated, Mg^{2+} -dependent ATPase activity. The calcium pump is activated by a calcium-binding protein called calmodulin.⁹

Amino acid transport in RBCs provides amino acids for synthesis of glutathione. In addition, amino acid transporters may be responsible for efflux of amino acids during reticulocyte maturation.²¹

Species vary in their permeability to glucose, with human RBCs being very permeable and pig RBCs being poorly permeable. Red blood cells of other domestic animals appear to be intermediate between these extremes.⁹ Glucose movement into RBCs is not regulated by insulin; rather facilitative glucose transporters mediate the passive diffusion of glucose into RBCs.¹⁸ Red blood cells from adult pigs lack a functional glucose transporter and, therefore, have limited ability to utilize glucose for energy.²

Red blood cell membranes from most animal species have a nucleoside transporter. Rabbit, pig, and human RBCs exhibit substantially more adenosine uptake than those of other species studied. Red blood cells from dogs exhibit more adenosine uptake than those of cats, goats, or cattle, and RBCs from horses and most sheep appear to be nearly impermeable to adenosine. While dog RBCs are permeable to adenosine, they are impermeable to inosine. Dog and cat RBCs exhibit adenine uptake and incorporation into nucleotides, but values are much lower than those of human, rabbit, or rodent RBCs.⁹

CARBOHYDRATE METABOLISM

Although substrates such as ribose, fructose, mannose, galactose, dihydroxyacetone, glyceraldehyde, adenosine, and inosine may be metabolized to some extent, depending on the species, glucose is the primary substrate for energy needs of RBCs from all species except the pig. Inosine appears to be the major substrate for pig RBCs. Its production by the liver is sufficient to meet RBC energy requirements.²⁵

Once glucose enters the cell, it is phosphorylated to glucose 6-phosphate (G6P) utilizing the hexokinase (HK) enzyme. The G6P is then metabolized through either the Embden-Meyerhof pathway (EMP) or the pentose phosphate pathway (PPP) as shown in Figure 21.1.

EMBDEN-MEYERHOF PATHWAY AND ATP PRODUCTION

A net of two molecules of ATP is produced for each molecule of glucose metabolized to two molecules of lactate in the EMP. Because mature RBCs lack mitochondria, the EMP is the only source of ATP production in these cells. Reactions catalyzed by HK, phosphofruktokinase (PFK), and pyruvate kinase (PK) appear to be rate-limiting steps in glycolysis, with the PFK reaction being most important under physiologic steady-state conditions.⁹

At physiologic pH values, high concentrations of inorganic phosphate (P_i) stimulate glycolysis through the EMP by reducing the ATP inhibition of PFK. Conversely, glycolysis is inhibited by short-term phosphate deficiency, primarily by decreasing intracellular P_i for glyceraldehyde phosphate dehydrogenase (GAPD). Decreased glycolytic rates result in decreased RBC ATP concentrations and hemolytic anemia in experimental dogs made severely hypophosphatemic by hyperalimentation. Hemolytic anemia associated with hypophosphatemia has also been reported in diabetic cats and a diabetic dog following insulin therapy, in a cat with hepatic lipidosis, and in postparturient cattle in which decreased RBC ATP concentrations have been measured.⁹

Because mature RBCs depend solely on anaerobic glycolysis for ATP generation, deficiencies of enzymes involved in glycolysis result in shortened RBC survival. Insufficient ATP generation in deficient RBCs can result in echinocyte formation. PK-deficient dogs and cats have mild to severe regenerative hemolytic anemia (see Chapter 28). PK-deficient dogs exhibit marked iron accumulation in the liver and die from liver failure or myelofibrosis.⁸ PFK-deficient dogs have compensated hemolytic anemia with sporadic episodes of intravascular hemolysis and hemoglobinuria (see Chapter 28).⁸ PFK-deficient dog RBCs are alkaline fragile, because 2,3-diphosphoglycerate (2,3DPG) concentration is decreased in these cells. A decrease in 2,3DPG, the major impermeant anion in dog RBCs, results in higher intracellular pH. Episodes of intravascular hemolysis occur when PFK-deficient dogs develop alkalemia secondary to hyperventilation.⁸

DIPHOSPHOGLYCERATE PATHWAY AND OXYGEN AFFINITY OF HEMOGLOBIN

Molecules of 1,3-diphosphoglycerate (1,3DPG), produced by the GAPD reaction, may be utilized by the phosphoglycerate kinase (PGK) reaction in the EMP or

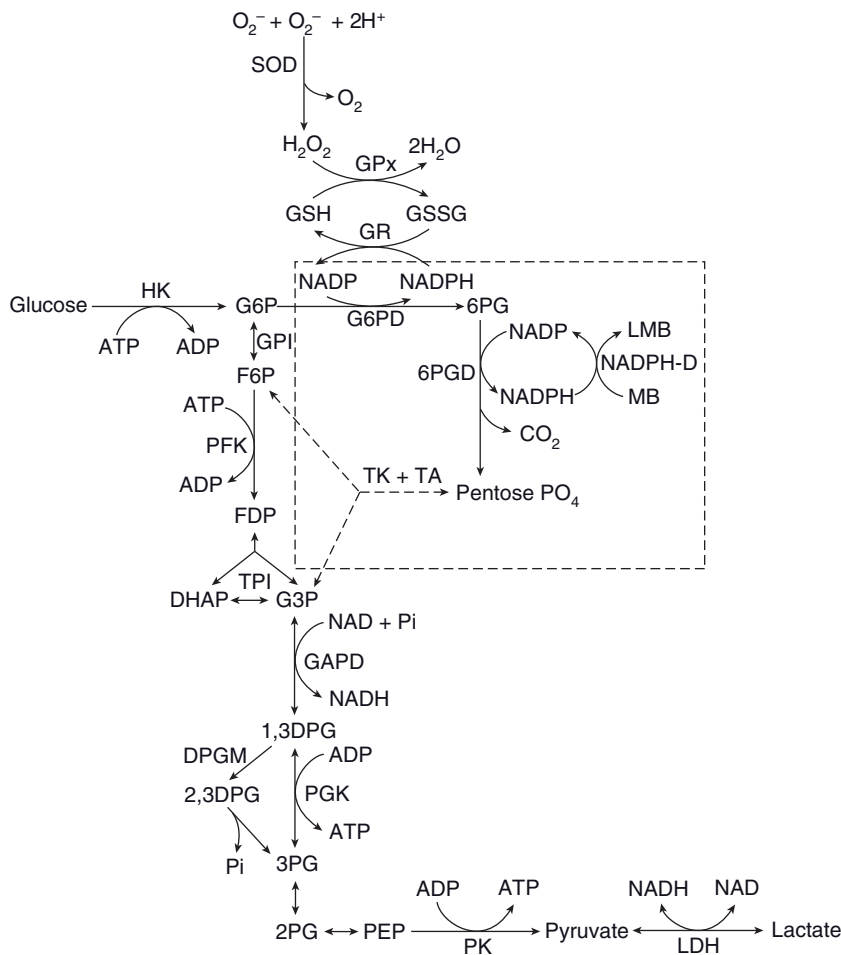


FIGURE 21.1 Metabolic pathways of the mature RBCs. HK, hexokinase; GPI, glucose phosphate isomerase; PFK, phosphofructokinase; TPI, triosephosphate isomerase; GAPD, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; MPGM, monophosphoglycerate mutase; DPGM, diphosphoglycerate mutase; PK, pyruvate kinase; G6PD, glucose-6-phosphate dehydrogenase; 6PGD, 6-phosphogluconate dehydrogenase; LDH, lactate dehydrogenase; LMB, leukomethylene blue; MB, methylene blue; GR, glutathione reductase; GPx, glutathione peroxidase; TK, transketolase; TA, transaldolase; GSSG, oxidized glutathione; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; FDP, fructose 1,6-diphosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate; 1,3-DPG, 1,3-diphosphoglycerate; 2,3-DPG, 2,3-diphosphoglycerate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; NAD, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NADPH-D, reduced nicotinamide adenine dinucleotide phosphate diaphorase; GSH, reduced glutathione; P_i , inorganic phosphate; SOD, superoxide dismutase. (Courtesy of John Harvey. This figure was published in *Clinical Biochemistry of Domestic Animals*, 6th ed., p. 196. ©Elsevier 2008.)

may be converted to 2,3DPG by the diphosphoglycerate mutase (DPGM) reaction (Fig. 21.1). 2,3DPG degradation to 3-phosphoglycerate (3PG) is catalyzed by diphosphoglycerate phosphatase activity (DPGP). The DPG pathway or shunt bypasses the ATP-generating PGK step in glycolysis; consequently, no net ATP is generated when glucose is metabolized through this pathway.

Red blood cells of dogs, horses, pigs, and man normally contain high concentrations of 2,3DPG, whereas those of cats and domestic ruminants have low concentrations. In RBCs from most mammalian species, 2,3DPG decreases the oxygen affinity of hemoglobin. When the oxygen affinity of hemoglobin is studied in hemolysates, removal of 2,3DPG and ATP (termed "stripping") from species with low 2,3DPG RBCs results in considerably lower oxygen affinities than removal from hemoglobin of species with high 2,3DPG RBCs. Because stripped hemoglobins from species with high 2,3DPG RBCs have high oxygen affinities, it appears that 2,3DPG is needed within RBCs of these species to maintain hemoglobin oxygen affinity within a physiologically useful range.⁹

The flow through the DPG pathway is regulated by the overall glycolytic rate. The formation of 2,3DPG is stimulated by increased phosphate concentration and increased pH. Hypoxic conditions stimulate 2,3DPG

synthesis primarily by inducing hyperventilation with resulting alkalosis. Conversely, acidosis and hypophosphatemia result in decreased 2,3DPG concentrations. PFK deficiency inhibits glycolysis above the DPG shunt and results in decreased 2,3DPG concentration. In contrast, the concentration of 2,3DPG is increased in dogs with PK deficiency because the metabolic block occurs below the DPG shunt.⁸

Erythrocyte 2,3DPG increases in anemic dogs and cats. The resultant decrease in hemoglobin oxygen affinity would seem to be beneficial in response to anemia in the dog. 2,3DPG concentration is much lower in cat RBCs than in dog erythrocytes, and cat hemoglobins are generally less responsive to 2,3DPG than dog hemoglobin; consequently, the physiologic significance of this increase in cats is unclear.⁹ Increased 2,3DPG has also been reported in RBCs from horses with hypoxic conditions.⁶ In the case of severe hypoxic hypoxemia the response might be detrimental, because hemoglobin cannot be fully saturated.¹²

OXIDANTS AND OXIDATIVE INJURY

Animals are exposed to low levels of oxidants in their environment and from normal metabolic processes in

the body. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are formed as products of normal cellular metabolism. At low to moderate concentrations, nitric oxide and superoxide (O_2^-) free radicals are involved in signal transductions between cells. When generated at higher concentrations in disease states, these free radicals (and even more potent oxidative metabolites that they produce) can overwhelm protective systems within the body, producing cellular injury and/or destruction.²²

Cumulative injury from these exposures may account for the normal aging and removal of circulating RBCs (see Chapter 22). Some metabolic disorders, including diabetes, inflammation, hyperthyroidism, neoplasia, RBC parasites, intense exercise, and ischemia/reperfusion can generate oxidants in sufficient amounts to result in increased oxidant injury and shortened erythrocyte lifespans, but the anemia (when present) is generally mild (see Chapter 36). A wide variety of drugs and environmental agents either exist as free radicals or can be converted to free radicals by cellular metabolic processes. These free radicals can be more damaging than ROS and RNS.⁹

Oxidants may damage RBC hemoglobin, enzymes (especially sulfhydryl groups), and membranes (especially polyunsaturated lipids). Methemoglobin forms when hemoglobin iron is oxidized from the ferrous to the ferric state. Methemoglobin is unable to bind oxygen, but its presence alone does not result in shortened erythrocyte lifespan.⁹

Heinz bodies are inclusions that form within RBCs following the oxidative denaturation of the globin portion of hemoglobin (see Chapter 36). They bind to the inner surface of RBC membranes, and their presence can result in premature RBC phagocytosis. Heinz bodies are frequently recognized in RBCs from cats because of the susceptibility of cat hemoglobin to form Heinz bodies, combined with a poor ability of the cat spleen to remove Heinz bodies from RBCs. Even normal cats may have low numbers of Heinz bodies (<5%).⁷

Oxidative membrane damage results in premature phagocytosis of injured RBCs. Oxidative injury to erythrocyte membranes may result in the adhesion of opposing areas of the cytoplasmic face of the RBC membrane and the formation of eccentrocytes and pyknotocytes. Eccentrocytes have primarily been recognized in dog and horse RBCs.⁷ Intravascular hemolysis may occur when severe membrane injury is present.

PENTOSE PHOSPHATE PATHWAY AND PROTECTION AGAINST OXIDANT

The PPP generates NADPH, the major source of reducing equivalents in the protection of RBCs against oxidative injury. Normally only about 5–13% of glucose metabolized by RBCs flows through the PPP (Fig. 21.1), but this flow can be accelerated markedly by oxidants.⁹ NADPH is needed to maintain glutathione and thioredoxin in the reduced state, and it is important in maintaining catalase in a functional form. Defects in the PPP can render RBCs susceptible to endogenous and exog-

enous oxidant injury. Glucose-6-phosphate dehydrogenase (G6PD) is the rate controlling enzyme in the PPP. A persistent hemolytic anemia with eccentrocytosis has been described in an American saddlebred colt with <1% of normal G6PD activity.²⁰

Glutathione is of central importance in the protection against oxidant injury. It is a tripeptide of glutamic acid, cysteine, and glycine that is synthesized *de novo* in RBCs of animals from constituent amino acids via two ATP-requiring reactions. Glutathione deficiency occurs in some sheep because of a deficiency of γ -glutamylcysteine synthetase, the first enzyme involved in glutathione synthesis, and in other sheep deficient in the amino acid transporter responsible for cysteine transport into RBCs, thereby limiting cysteine uptake and restricting glutathione synthesis.⁹

Reduced glutathione (GSH) has a highly reactive sulfhydryl group that may act nonenzymatically as a free radical acceptor to counteract oxidant damage. GSH also functions as an electron donor in various reductive enzyme reactions including glutathione peroxidase (GPx), phospholipid hydroperoxide glutathione peroxidase, glutathione S-transferase, and glutaredoxin.⁹

Oxidized glutathione (GSSG) produced by these reactions is reduced back to GSH by using the flavin adenine dinucleotide (FAD)-dependent glutathione reduction (GR) reaction that requires NADPH. Horses with RBC FAD deficiency have markedly reduced GR activity, decreased GSH concentration, and prominent eccentrocytosis.¹⁰

Superoxide dismutase (SOD) within RBCs is a copper- and zinc-containing enzyme that promotes the dismutation of two O_2^- molecules to H_2O_2 and oxygen (Fig. 21.1). The importance of SOD as an oxidant defense in RBCs is unclear. Although generally considered protective, SOD may actually increase oxidant injury in conditions where H_2O_2 catabolism is compromised.⁹

GPx catalyzes the conversion of H_2O_2 to water. It also catalyzes the reduction of fatty acid hydroperoxides, and 1-monoacylglycerol hydroperoxides. Another GPx in RBCs termed phospholipid hydroperoxide glutathione peroxidase participates in the reduction of more complex phospholipid hydroperoxides using GSH.

Catalase is a heme-containing enzyme that also destroys H_2O_2 by conversion to water and oxygen. Except for dogs, mammalian RBCs generally have high catalase activities.⁹

Recent studies suggest that peroxiredoxins may be more important in protecting against H_2O_2 than GPx or catalase.¹⁶ These multifunctional SH-dependent enzymes reduce H_2O_2 and alkyl hydroperoxides to water and alcohol, respectively. Oxidized peroxiredoxins are regenerated using reduced thioredoxin, and oxidized thioredoxin is reduced by NADPH using thioredoxin reductase.¹⁴ Thioredoxin may also be important in the direct scavenging of free radicals. Thioredoxin and glutaredoxin are small proteins with two closely associated cysteines in their active sites. The reduced forms of these proteins interact with and reduce intramolecular protein disulfides that form in oxidatively damaged proteins.¹⁵ Oxidized glutaredoxin is reduced by GSH.

Selenium is incorporated as selenocysteine at the active site of a wide range of selenoproteins, including GPx, phospholipid hydroperoxide glutathione peroxidase, and thioredoxin reductase in RBCs.¹ Heinz body hemolytic anemia has been reported in selenium deficient cattle grazing on St. Augustine grass.¹⁹

Vitamin E (α -tocopherol) is lipid-soluble and the most important antioxidant in the cell membrane. It donates reducing equivalents to lipid peroxyl radicals, converting them to less toxic lipid hydroperoxides.²² Oxidized vitamin E can be reduced by ascorbate, presumably at the aqueous-lipid interface of the lipid bilayer.¹⁷ Vitamin E deficiency increases the susceptibility of erythrocytes to peroxidative hemolysis.

Ascorbate donates one or two electrons to a variety of oxidants, including oxygen free radicals and peroxides. Dehydroascorbate can be reduced to ascorbate nonenzymatically by GSH and enzymatically via the glutaredoxin reaction, by a separate GSH-dependent dehydroascorbate reductase enzyme, and by the thioredoxin reductase reaction.^{17,23}

METHEMOGLOBIN FORMATION AND REDUCTION

About 3% of hemoglobin within RBCs is oxidized to methemoglobin each day in normal animals; however, methemoglobin usually accounts for less than 1% of total hemoglobin, because it is constantly reduced back to hemoglobin by a cytochrome-b₅-reductase (Cb₅R) enzyme reaction present in RBCs.⁹ In this reaction, electrons are transferred from NADH generated in glycolysis to ferricytochrome-b₅ using FAD as the enzyme-bound prosthetic group; then the resulting ferrocycytochrome-b₅ reduces methemoglobin nonenzymatically to hemoglobin. Methemoglobin reduction is more corrective than protective. Methemoglobin forms at much higher levels in the presence of oxidative compounds. An inherited deficiency in Cb₅R in dogs and cats results in persistent methemoglobinemia with minimal or no clinical signs.⁸ Methemoglobinemia also occurs in horses that have decreased Cb₅R activity secondary to RBC FAD deficiency.¹⁰

Methylene blue (MB) is used to treat toxic methemoglobinemia. It causes methemoglobin to be reduced faster than occurs by the relatively slow NADH-dependent methemoglobin reductase reaction. MB is reduced to leukomethylene blue (LMB) by NADPH-dependent diaphorase activity (Fig. 21.1), and LMB reacts spontaneously with methemoglobin, reducing it to hemoglobin and regenerating MB.⁹

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Erythrokinetics and Erythrocyte Destruction

JOHN A. CHRISTIAN

Reticulocyte Response

- Reticulocyte Maturation
 - Reticulocyte Identification and Counting
- ## RBC Lifespan
- Species Variation
 - Methods
- ## Mechanisms of RBC Clearance
- Methods of Approach
 - Indirect Aging Markers

RBC Clearance Mechanisms

- Rheological compromise
 - IgG binding
 - Complement
 - Oxidation of proteins
 - Other potential clearance mechanisms
- ## RBC Destruction

Acronyms and Abbreviations

Bc, conjugated bilirubin; BRBC, biotinylated RBCs; Bu, unconjugated bilirubin; DGC, density gradient centrifugation; ECM, extracellular matrix; Hct, hematocrit; Hgb, hemoglobin; Hsp 70, heat shock protein 70; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; NMB, new methylene blue; PS, phosphatidylserine; RBC, red blood cell; RNA, ribonucleic acid; RPI, reticulocyte production index; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SOD, superoxide dismutase; TfR, transferrin receptor.

The anuclear red blood cell (RBC) lifespan is characterized by three phases. A brief reticulocyte maturation period features rapid changes supported by a high rate of metabolism and synthetic function. The mature cell phase involves months of precise functioning under rigorous circulatory conditions. In the final, relatively brief senescence phase RBCs incur age-associated lesions involving rheological fitness, antioxidant defenses, and membrane structure. Less viable cells are removed from the circulation predominantly by cells of the mononuclear phagocyte system. The production activities of reticulocytes, their maturation and release, and processes of erythrocyte aging and destruction are covered in this chapter.

RETICULOCYTE RESPONSE

Reticulocyte Maturation

A reticulocyte is produced when a late stage nucleated erythroid precursor (metarubricyte) expels its nucleus. The reticulocyte then develops into a mature circulating RBC in 3–4 days, but at inception it is in many respects

quite distinctive from the mature cell.^{28,67} In contrast to the relatively uniform biconcave shape of mature cells in many species, reticulocytes are larger, round to lobulated cells with considerably more surface area and less cytoplasm. They have medium to large irregular invaginations and small superficial clathrin-coated pits used for endocytic activities.³³ Transmembrane receptors, transporter proteins, clathrin-coated pits and other transport/endocytic functions are actively providing essential building blocks (amino acids, iron, etc.) for synthesis of proteins and other components. At this stage 20–30% of total Hb must still be produced.^{27,67} These production activities require an actively synthesizing cytoplasm complete with ribosomes, polyribosomes, RNA and mitochondria, all of which will be removed within the next 3–4 days.

Cell maturation requires a degradation and clean-up process for components that will not be needed in mature cells. Proteolytic enzymes and RNAases, such as pyrimidine 5' nucleotidase, are responsible for degradation of various proteins, ribosomes, and RNA. Low numbers of lysosomes can facilitate limited degradation. A specific 15-lipoxygenase has been shown to initiate degeneration of mitochondria via programmed cell

death (mitoptosis). The enzyme works through production of reactive oxygen species and also binds to endoplasmic reticulum, Golgi apparatus, and peroxisomes.²⁷

Exosomes play a major role in the remodeling and extrusion process.^{3,27} Briefly, exosomes are formed by endocytic invaginations of membrane that are assembled into multivesicular exosomes. These structures then fuse with the internal cell surface where small (~60nm) membrane bound vesicles (exosomes) are released. Some estimates suggest that exosomes account for most of the membrane loss associated with maturation, about 30% in rats. The membranes of released exosomes have features (e.g. phosphatidylserine [PS] exposure) that allow recognition and removal by macrophages as apoptotic bodies, avoiding the incitation of inflammation or possibly an autoimmune response.³ Cell components shown to be selectively enriched in exosomes include receptors for transferrin (TfR), fibronectin, and insulin, and transporters for glucose, nucleosides, and amino acids.²⁷ Membrane Na-K ATPase is also removed from reticulocytes of carnivores that switch to low K⁺:high Na⁺ cytoplasm in mature RBCs. Heat shock protein-70 (Hsp-70), a chaperone protein, has been strongly implicated in the enrichment of TfR and probably other membrane proteins into exosomes.^{26,27}

Maturing reticulocytes are found primarily in the bone marrow where they can be sheltered until better prepared for circulatory stresses. In vitro studies indicate that the youngest reticulocytes have significantly decreased membrane stability and deformability by as much as 90% compared to later stage reticulocytes and mature RBCs.^{7,76,85} As cells mature several changes prepare the cell for release from the marrow into the circulating pool. The membrane receptors that tether reticulocytes to fibronectin and thrombospondin in the marrow extracellular matrix (ECM) are gradually lost, apparently through transfer to exosomes. Exosome release also reduces membrane surface area and aids in remodeling of the membrane. Coupled with cytoskeleton maturation and stability, this leads to smaller size and improved deformability. Surface sialic acids are decreased, which reduces negative charge repulsion between reticulocytes and endothelial cells. Subsequent contact with adventitial and endothelial cells appears to initiate thinning of the adventitia and separation between endothelial cell junctions that allows diapedesis of RBCs through pores into circulation.² In species where some reticulocytes are allowed to circulate in health, the cells appear to be preferentially sequestered for a period in the spleen where reticulocyte “ripening” allows for further hemoglobinization, membrane remodeling, and cytoskeletal stabilization. Splenic retention is apparently mediated through residual fibronectin receptors on reticulocytes.⁶⁰

Reticulocyte Identification and Counting

Reticulocytes are identified based on their appearance when exposed to supravital stains (new methylene

blue, brilliant cresyl blue, etc.). The dye causes precipitation of ribosomal RNA and organelles into a reticulum that is recognized as clumped aggregates of dark blue material (aggregate reticulocytes) or as two or more small punctate structures (punctate reticulocytes), depending on the amount of RNA present. With Romanowsky-type stains (Wright-Giemsa) no precipitate is formed. RNA remains dispersed and confers a uniform light blue color to the cells that are called polychromatophils. Polychromatophils correspond to aggregate reticulocytes and, although less sensitive, are useful on routine peripheral blood smears for characterizing marrow responsiveness to anemia.

The maturation time for aggregate and punctate cells and the maturation stage at which reticulocytes are normally released from the marrow vary with species. Dogs normally release aggregate reticulocytes from the bone marrow (usually $\leq 1.0\%$), which then complete maturation in roughly 24 hours. Punctate forms are short lived and are seen in insignificant numbers. Cats normally release low numbers of aggregate reticulocytes in health ($< 0.4\%$). These have a short circulating maturation time of about 12 hours to become punctate cells which then circulate for 10–12 days and may represent 10% of RBCs in health.^{16,82} Horses do not release reticulocytes from bone marrow in health and only rarely in regenerative anemia. However, RNA-containing equine RBCs can be recognized by automated technology, which might improve sensitivity in assessing equine anemia.¹⁴ Reticulocytes are typically not seen in the peripheral blood of cattle, sheep, or goats except in response to anemia. Healthy laboratory animal species that have shorter RBC lifespans and higher rates of erythropoiesis typically release reticulocytes at rates equal to or greater than are seen in dogs. Normal reticulocyte information for a wide range of species has been tabulated (see Section IX).²²

Cat aggregate reticulocyte counts and polychromatophilic cell proportions are lower than seen in healthy dogs or dogs with similar degrees of anemia, suggesting inherently inferior responsiveness to anemia. This species comparison may be misleading because bone marrow regenerative robustness is reportedly greatest in the cat among common domestic species.⁸² Accordingly, the feline punctate reticulocyte counts may be quite high, exceeding $4 \times 10^6/\mu\text{L}$. It appears that the rapid aggregate cell maturation in circulation masks the overall intensity of regeneration in cats. Historically, punctate reticulocyte counts in cats have been omitted in many laboratories. Because aggregate cells are so short lived and elevated punctate counts in cats do reflect a recent regenerative response, reporting of both types is advisable. Omission of punctate counts may lead to misclassification of anemia.

Although several automated analyzers capable of providing accurate and sensitive reticulocyte counts are available, manual methods are still used (see Chapter 136). The reticulocyte percent can easily be misinterpreted because the percentage depends not only on marrow production, but on the number of mature cells in circulation. The same number of

reticulocytes per μL of blood will represent a very different percentage when accompanied by normal numbers of mature cells (Hct 45) versus greatly reduced mature RBC numbers (Hct 15). To correct for this, the reticulocyte percent is multiplied by the total RBC count to obtain the “absolute reticulocyte count” (reticulocytes/ μL). Healthy dogs and cats generally have $\leq 80,000/\mu\text{L}$ and $\leq 60,000/\mu\text{L}$ absolute reticulocyte counts, respectively. If the RBC count is not available, the Hct can be used to calculate a “corrected reticulocyte percent” which makes a similar adjustment for the effect of relative RBC mass on reticulocyte percent. This value is obtained by multiplying the patient reticulocyte percent times the patient Hct/normal Hct. The normal Hct value is typically 45 for dogs and 37 for cats, values which roughly represent the middle of their respective species reference ranges.

As severity of anemia increases, reticulocytes tend to be released earlier in the marrow maturation process (i.e. shift reticulocytes). Because more immature reticulocytes circulate longer, reticulocyte counts will be higher but this reflects prolonged circulation time rather than increased production. This overestimation of marrow responsiveness can be corrected by calculating the “reticulocyte production index” (RPI). In humans and presumably in dogs, it is estimated that the reticulocyte circulating maturation time increases by 0.5 days for each 10-unit decrease in Hct. At Hct 45 the normal maturation time is 1 day. Therefore, the maturation time at Hct 35 is 1.5 days, at Hct 25 it is 2.0 days, and at Hct 15 it is 2.5 days. The RPI is calculated by dividing the corrected reticulocyte percentage (or absolute reticulocyte count) by the above maturation factor. For example, if a patient has a Hct of 25 (maturation time = 2 days) and a corrected reticulocyte percentage of 3, the RPI is $3.0/2 = 1.5$. This substantially reduces the estimation of marrow responsiveness. The RPI adjustment is not recommended in other species due to lack of information on the impact of various Hct levels on circulating maturation times.

RBC LIFESPAN

Species Variation

After release from marrow, RBCs circulate throughout the vascular system thousands of times before being removed, disassembled, and recycled by macrophages. The RBC lifespan varies characteristically with species and the differences are at least partially explained by different metabolic rates. Comparative studies reveal that RBC lifespan correlates positively with species longevity (years) and with body mass. That is, larger mammals tend to live longer and have longer RBC lifespans when compared to smaller species. Smaller species have higher rates of oxidative metabolism and presumably accrue oxidative injury at higher rates, reducing both longevity and RBC lifespan. This relationship is illustrated by comparison among common mammalian species where the RBC lifespan is approxi-

mately 140–145 days for horses, 130 days for cattle, 115 days for goats, 100–115 days for dogs, 73 days for cats and 43 days for mice.⁷⁴

Other experimental evidence suggests that RBC lifespan in health may be influenced by RBC antioxidant status. Kurata et al. found that RBC potential lifespan in several mammalian species ($n = 10$) correlated significantly with the RBC levels of superoxide dismutase (SOD), glutathione peroxidase, and glutathione.⁴⁵ Additionally, RBC viability may be negatively influenced by corporal aging, at least in small laboratory animals. RBCs from old rats are reported to have lower quantities of several antioxidant enzymes compared to young rat RBCs.³⁰ Reticulocytes from old rats have lower SOD activity, and increased quantities of catalytically inactive SOD, suggesting an age-associated defect in erythropoiesis.³⁰ These differences are associated with altered RBC survival as evidenced by a reported 40–60% shorter RBC lifespan in old mice and rats versus young animals.^{1,29} Although limited information is available for larger animals, a similar pattern of shorter RBC lifespan with age has been suggested for greyhounds.⁵⁹

Not all circulating RBCs are removed exclusively based on cell age (senescence-based clearance). In most species of mammals, a variable proportion of RBCs leave the circulation independently of cell age (random clearance). Although the mechanism for random clearance is speculative, the degree of random clearance does appear to correlate positively with metabolic rate.⁷⁴ Higher rates of random loss are, therefore found in smaller species (e.g. rabbits, rodents) and, because senescent and random clearance cannot be readily distinguished, random loss confounds studies attempting to characterize mechanisms for senescence-based clearance.

Methods

Red blood cell lifespan studies have traditionally used radioisotopes and such studies usually take one of two forms: random or cohort labeling.⁴⁶ A random study labels an aliquot of RBCs having an age distribution similar to that normally found in circulation. As these RBCs circulate, the steady decrease in survival (as measured by a decrease in radioactivity) is ideally a result of normal RBC turnover. For instance, in humans and dogs, where ~1% of RBCs are lost each day, the radioactivity would decrease by 1% per day. Isotopes like ⁵¹Cr and ¹⁴C-cyanate are typically used for random RBC studies. On the other hand, cohort studies attempt to label RBCs of a similar age so that, barring random RBC loss, radioactivity in circulation remains constant until the cohort becomes senescent. At that time radioactivity decreases rapidly as the cohort of old RBCs is removed from circulation. Isotopes such as ¹⁵N-glycine, ⁵⁵Fe, and ⁵⁹Fe that are incorporated into developing RBCs are commonly used for cohort studies.

A thorough knowledge of any probe used for measuring RBC lifespan is essential to obtaining accurate

results. An ideal probe will not be: toxic to cells or the host, immunogenic, eluted from the cells before RBC clearance, or reutilized in making new RBCs (recycled). Corrective formulas may help adjust for a failure to meet these criteria, but corrective formulas sometimes must be species-specific. For instance, elution of ^{51}Cr from RBCs in circulation renders determination of RBC survival complicated.³¹ The degree of elution can vary considerably between species.⁴⁸ Therefore, formulas correcting for elution loss in humans are unlikely to be applicable in other species. Use of iron radioisotopes to measure RBC lifespan is problematic because upon destruction of labeled RBCs the isotope is recycled into the Hb of new cells.^{23,70} Corrections are required because recycling increases values for RBC survival. The ^{14}C -cyanate label is a better probe in that it can be used at non-toxic levels, it does not elute from cells, and it is not reused.^{6,32,47}

Several nonradioactive, random labeling probes have been developed that increase safety and convenience of measuring RBC lifespan. Most are fluorescence-activated probes that allow detection of the proportion of labeled RBCs over time by flow cytometry.^{9,35,64} These probes require similar validation as isotopic methods. Biotinylation of RBCs has been one of the most widely used approaches. Labeled RBCs are detected based on affinity to avidin-conjugated fluorescent probes (e.g. fluorescein derivatives).

MECHANISMS OF RBC CLEARANCE

Methods of Approach

Compared to direct measurement of RBC lifespan, isolation of unequivocally old RBCs for analytical studies has been a formidable task. Historically, isolation of dense RBCs by density gradient centrifugation (DGC) has been the most popular method for collecting presumably senescent RBCs. This approach was first supported by human studies suggesting that as radiolabeled cohorts of RBC aged, fractionation on density gradients resulted in increasing enrichment of labeled cells in more dense centrifugation fractions.⁵ It was also reported that the most dense fraction of human RBCs had very short survival upon autologous reinfusion.⁷² However, many different separation media and methodologies have been used without standardization and frequently without adequate validation.¹⁵ This has led to inconsistent and often contradictory results so that controversy has plagued these studies.

To bypass these controversies, unique approaches for *in vivo* aging of RBCs were developed in animal models. These studies use defined RBC populations that circulate for specific time intervals before RBCs are isolated and analyzed. The advantage of these “*in vivo*” aging approaches is that RBCs are selected based on known time in circulation rather than on a controversial trait that is assumed to correlate with cell age.

The first approach involved serial hypertransfusion of laboratory animals resulting in polycythemic shut-

down of erythropoiesis over the lifespan of circulating RBCs. At specific intervals of the normal RBC lifespan, RBCs of known circulatory age range are then collected and analyzed.^{25,56,87}

Using a different approach, RBC senescence in several species has been evaluated by covalently linking biotin to RBC membranes.^{10,71} After defined periods of circulation, biotinylated RBCs can be isolated from other cells based on affinity to an avidin support, and subsequently analyzed. The purity of biotinylated cells can be determined using a secondary avidin label conjugated to a fluorescent probe.

These approaches have been used to test if increasing RBC density is a significant component of the aging process in various species. Hypertransfused rat RBCs did show increasing cell density (i.e. MCHC) early in the RBC lifespan but not late, suggesting mainly a maturation effect.²⁵ Hypertransfused mice showed minimal, age-associated changes in RBC density profiles.^{56,57} Rabbit biotinylated RBCs did show some senescence-associated enrichment in the most dense RBC fractions generated on Percoll media. But these old biotinylated RBCs only comprised about one-third of the dense RBCs, indicating that density gradient-derived RBCs were poorly representative of the oldest cells.¹⁷ Conversely, the canine biotinylation model has demonstrated quantitatively that the 1% most dense RBCs fractionated on arabinogalactan gradients are at least moderately enriched for old RBCs.¹¹ Over 75% of these dense cells were between 86 and 115 days old with a mean cell age of approximately 101 days (RBC lifespan 100–115 days). Although clearly enriched for old cells, it is still unclear if the most critical changes occurring in the last few days of RBC survival are well represented in this population.

Indirect Aging Markers

Controversy over the analysis of dense cells as old cells has also led to the search for cellular aging markers that could be used to verify that the isolated RBCs are actually the oldest RBCs. These markers may not be associated with RBC clearance *per se*, but are intended to simply validate that the RBCs being studied are among the oldest cells. Many markers have been proposed, but most have eventually been shown to reflect maturation rather than aging (e.g. insulin receptor, pyrimidine 5 nucleotidase, ATP levels, glycolytic enzymes, etc.) A couple of candidates remain as valid markers of cell age.

The best characterized aging marker involves changes in RBC membrane protein 4.1. In the mouse hypertransfusion model a relatively constant change in the ratio of membrane skeletal proteins 4.1a and 4.1b was observed by electrophoresis (SDS-PAGE) throughout the lifespan.⁵⁷ Although total band 4.1 remained relatively constant, the proportion contributed by 4.1a went progressively from 8% at mean cell age of 4 days to 54% at mean cell age of 57.5 days (mouse RBC lifespan = 60 days). In control cells at mean cell age of 30 days, band 4.1a accounted for 30% of total band 4.1.

With the exception of the cat, this phenomenon is observed in humans and in all of the common domestic mammals.^{37,62} The cause of this change in protein ratios has been defined. In the mouse, protein 4.1b is transcribed and inserted into the membrane at an early stage of erythroid development.³⁷ Protein 4.1a is then formed by post-translational modification of 4.1b. A study using human RBCs demonstrated that the conversion of 4.1b to 4.1a results from deamidation of asparagine 502.³⁸ This change is responsible for the apparent molecular weight shift seen on electrophoresis. The change is probably not a factor in removal of aged RBCs; however, the ratio has been used to confirm the relative increase in age of the densest RBCs isolated on arabinogalactan⁴¹ and on Percoll⁵⁴ media.

Glycated hemoglobin (HbA1c) also has been proposed as a marker of RBC aging. HbA1c is formed by the irreversible, nonenzymatic binding of glucose to hemoglobin (Hgb), which is dependent on plasma glucose concentration and time of exposure (i.e. RBC lifespan).²⁴ Its primary use has been in monitoring time-averaged glycemic control in diabetics. However, if plasma glucose concentration is held constant, variations in HbA1c should be a function of RBC age. There is evidence that HbA1c levels increase with increasing RBC density.²¹

RBC Clearance Mechanisms

The mechanisms of RBC clearance may be multiple and the importance of each may vary with species. It appears that the best supported clearance mechanisms are designed to remove old RBCs well before significant decrement in individual cells might compromise overall RBC functionality. Thus, redundancy in mechanisms to ensure timely clearance would not be surprising.

Rheological Compromise

Based on evidence that cell density and cell age were positively correlated,⁵ early RBC aging studies concentrated heavily on identifying "worn out" cells with severely compromised rheological abilities. Since then, some well-designed comparative studies using *in vivo* aged rabbit biotinylated RBCs, hypertransfused mouse RBCs, and human dense RBCs have confirmed that some rheological compromise accompanies cell aging.^{54,77} But the magnitude and significance of these changes in explaining RBC clearance remain equivocal. Although mild decreases in deformability were observed, fairly constant surface area to volume ratios (sphericity) and a slight decrease in diameter offset that change and convincing support that the RBCs would be compromised in traversing sinusoids was lacking.

In contrast to age-related rheological changes as a primary clearance mechanism, much study has been directed at finding sensitive and specific signals that trigger clearance of aging RBCs long before they become effete. Both immunologic and nonimmunologic pathways of RBC clearance have been identified.

IgG Binding

Autologous immunoglobulin binding to the most dense human RBCs and increased phagocytosis of these RBCs by autologous macrophages was first described by Kay.⁴² Fab dependent IgG binding and phagocytosis suggested the involvement of specific receptor recognition by the IgG.⁴³ Supporting Kay's findings, several other density gradient centrifugation based studies have also reported increased immunoglobulin binding in the most dense RBCs.^{29,41,53} Furthermore, using the mouse hypertransfusion model of *in vivo* aging, autologous IgG was identified only in the oldest RBCs and was associated with *in vitro* phagocytosis.⁶⁶ Increased IgG binding to dog biotinylated RBCs ≥ 104 days old has been demonstrated, although the ability of the IgG binding to mediate phagocytosis was not tested.⁸ By contrast, *in vivo* aged rabbit biotinylated RBCs failed to show terminal increases in IgG binding,¹⁸ which further highlights the need for caution in extrapolating information between species.

The identity of the epitope responsible for antibody binding to senescent RBCs has been controversial, although it is not unreasonable to consider that more than one epitope may develop and that this could vary between species. The anion transport protein (band 3 on SDS-PAGE) has received the most support as an important site, although other sites such as glycophorin A and aminophospholipids, have also been proposed. Band 3 reportedly becomes antigenic through either proteolytic cleavage revealing a 62 kDa immunoreactive fragment, or by horizontal redistribution of the transmembrane protein into immunoreactive clusters. Exposure of the cryptic 62 kDa antigen by proteolysis is proposed to occur as a consequence of cumulative oxidative injury.⁴⁴ Interestingly, cat RBCs exposed to activated neutrophils show evidence of proteolysis and increased IgG binding.⁷⁹⁻⁸¹ The changes, that appear to contribute to accelerated clearance of RBCs in the anemia of inflammatory disease, ensued from a combination of oxidation and serine protease activity. Lower level, cumulative injury could conceivably play a role in RBC injury and clearance under normal homeostasis, as described Kay et al.⁴⁴

The role of band 3 clustering in initiating autologous antibody binding has been well documented in senescent and diseased RBCs.^{40,41,49,51,55,75,78,86} It is theorized that unclustered band 3 does not sustain antibody binding because the senescent cell IgG binds weakly and requires a two-point attachment to form a stable association. Normally the distance between band 3 molecules is too great for the binding of both Fab portions of IgG. Clustering of band 3 can be accomplished by several methods, including binding of denatured Hgb (i.e. hemichromes or Heinz bodies) at the cytoplasmic domain of band 3 or by chemical agents such as zinc, mellitin, and acridine orange.^{12,73,75} Oxidized, denatured Hgb has a specific high affinity binding site on the cytoplasmic domain of band 3. This binding leads to a horizontal redistribution of band 3 into a clustered arrangement that is then recognized on the external

surface by autologous antibodies. This pathway illustrates how an early intracellular compromise in maintaining reduced Hgb could initiate a morphologic change on the external cell surface leading to opsonization and phagocytosis. Membrane aggregates showing enrichment and co-localization for hemichromes, band 3, and IgG have been identified in a variety of conditions, including dense human RBCs, sickle RBCs, and thalassemic RBCs.^{40,41,78,86} The pathway of Hgb denaturation leading to band 3 clustering and opsonization is at least partially supported by *in vivo* RBC aging studies. Hypertransfused mouse RBCs and dog biotinylated RBCs both show increased membrane-bound Hgb and increased IgG binding late in the RBC lifespan, although these changes have not yet been specifically co-localized to band 3 clusters.^{56,63} Clustering of band 3 using chemical agents (acridine orange, Zn²⁺, melittin) also causes autologous IgG binding apart from hemichrome formation, supporting the premise that it is the clustering of band 3 and not the oxidation process *per se* that creates the signal for IgG binding.^{49,73} Removal of the clustering agents in each case, led to reversal of the clustered distribution and elution of antibodies.

Opsonization of RBCs secondary to band 3 clustering may play a notable role in the pathogenesis of several hemolytic anemias of significant importance to veterinary medicine. Heinz body hemolytic anemia occurs in several species as a result of exogenous intoxicants and, in the cat, associated with certain metabolic diseases. Because Heinz bodies bind to the cytoplasmic domain of band 3, subsequent clustering and opsonization may lead to an immune-mediated component in clearance of these cells.

Zinc intoxication in dogs is frequently associated with severe hemolytic anemia. Strong regenerative responses with spherocytosis and inflammatory leukograms have led clinicians to initially diagnose this as immune-mediated hemolytic anemia. Given the clear role of zinc in clustering of band 3 with subsequent opsonization, this toxicity is likely an immune-mediated process. Although initially counterintuitive, this condition is consistently reported as Coombs' test negative. But this is very consistent with the reversible nature of band 3 clustering by zinc. RBCs are washed before running the assay. Washing would remove zinc, leading to dispersion of the band 3 clusters and elution of the antibodies. Finally, melittin in bee venom is another band 3 clustering agent. Although other elements in bee venom (e.g. phospholipase A₂) are clearly important in the destruction of RBCs, the potential for an immunologic component secondary to band 3 clustering should also be considered.

Complement

Another factor likely to be important in the efficient removal of IgG-opsonized RBCs is amplification of the signal by complement. Turrini and coworkers showed that when band 3 in human RBCs was clustered using ZnCl₂, a sequence of IgG binding, complement binding, and *in vitro* phagocytosis followed.⁷³ Phagocytosis was

maximal in the presence of clustered band 3, IgG, and complement. Inactivation of complement reduced phagocytosis by 80% which demonstrated IgG's weak capacity to initiate phagocytosis alone and emphasized complement's importance in amplification. However, phagocytosis was virtually eliminated if IgG or band 3 clustering was removed, showing the absolute requirement for these steps. This work complemented previous reports describing the presence of C3b-IgG complexes on oxidatively stressed⁵⁰ and density-gradient derived "senescent" human RBCs.⁵² Interestingly, only low numbers of IgG (an increase of <20 IgG/RBC) were needed to stimulate significant complement deposition.⁵⁰ Quantitative studies on antibody binding to dense RBCs have generally reported an increase of only a few hundred IgG per RBC.^{41,43} With amplification, this degree of antibody binding should be a more than adequate signal to initiate phagocytosis.

Oxidation of Proteins

Several indications of compromised antioxidant capacity have been observed in dense and *in vivo* aged RBCs from various species. A few examples include decreases in total free thiol and reduced glutathione, glutathione reductase activity, and increases in methemoglobin, membrane bound hemichrome, and lipid peroxidation byproducts.^{1,36,39,41} Although there has been controversy over the significance of reported compromises in antioxidant defenses,¹³ the presence of irreducible denatured hemoglobin bound to membranes is difficult to dismiss as clear evidence of antioxidant decline. At least two different pathways have been proposed whereby interaction of oxidized Hgb with the membrane might lead to RBC removal (see Chapter 36). One is hemoglobin-spectrin cross-linking leading to increased membrane rigidity, decreased deformability and/or stimulation of membrane proteolysis with subsequent breakdown in membrane integrity.^{68,69} The other pathway is as described earlier whereby oxidized Hgb binds to and clusters band 3 protein leading to opsonization and clearance.

Other Potential Clearance Mechanisms

A number of other lesions in senescent RBCs have been proposed as playing a role in clearance. With evidence for some decline in antioxidant capabilities there is also a risk of lipid peroxidation that could facilitate RBC removal by either compromising deformability⁶¹ or triggering recognition by macrophages.⁶⁵ The latter is suggested to occur through exposure of phosphatidylserine on the outer leaflet of the lipid bilayer. Whether secondary to oxidative injury, depletion of energy, or other mechanisms, there is ample evidence that the presentation of negatively charged phosphatidylserine on the outer layer of RBCs leads to phagocytosis by macrophages.⁶⁵ In the rabbit biotinylation model, older RBCs were found to expose more phosphatidylserine than younger RBCs and the increased exposure correlated well with senescence-based removal, but not with

random removal of RBCs.⁴ This may well be an important mechanism for clearance of senescent RBCs, particularly in the rabbit where IgG binding does not appear to be a significant factor in the clearance of old RBCs.¹⁸ Recognition of phosphatidylserine exposure on RBCs by macrophages can be at least partially mediated by lactadherin, a phosphatidylserine binding glycoprotein produced by macrophages.¹⁹

RBC DESTRUCTION

The primary organs of RBC clearance are the spleen, liver, and bone marrow. Phagocytosed RBCs are digested within macrophage phagolysosomes, and hemoglobin is dissociated into heme and globin. Globin is further digested and resulting amino acids are reutilized. Heme breakdown produces iron, biliverdin and small amounts of carbon monoxide. Heme oxygenase-1 (HO-1) is the rate-limiting enzyme in heme degradation and is responsible for release of iron. Heme iron may be stored in macrophages or exported to the circulation by ferroportin.^{20,58} Biliverdin is converted within the macrophage to bilirubin (unconjugated, Bu) that is relatively water insoluble. As bilirubin leaves the cell it attaches noncovalently to albumin to improve solubility and to be transported to the liver. Bu uptake by hepatocytes and conjugation with glucuronides (most species) produces the water-soluble conjugated bilirubin (Bc) that is excreted by active transport into biliary canaliculi for excretion in the intestines. There Bc is broken down to urobilinogen which is either reabsorbed or is converted to stercobilinogen, which contributes to the dark brown color of feces, and excreted. Reabsorbed urobilinogen is either taken up by hepatocytes and excreted in the bile or enters general circulation and is excreted in the urine.

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Erythrocyte Morphology

ANNE M. BARGER

Normal Erythrocyte Morphology and Distribution

Variation in Erythrocyte Morphology

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Cell Size

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Acronyms and Abbreviations

DIC, diffuse intravascular coagulation; FAD, flavin adenine dinucleotide; GN, glomerulonephritis; HJ, Howell-Jolly body; IMHA, immune-mediated hemolytic anemia; MCV, mean corpuscular volume; NMB, new methylene blue; nRBC, nucleated red blood cell; RBC, red blood cell.

Evaluation of red blood cell (RBC) morphology is a critical step in thorough blood smear evaluation and can aid in identifying many different metabolic disorders, indicate oxidative damage or help localize a disease process. There are four features of red blood cell morphology that should be evaluated: color, size, shape and inclusions.

NORMAL ERYTHROCYTE MORPHOLOGY AND DISTRIBUTION

The typical RBC shape for multiple veterinary species is the disc or biconcave disc (i.e. discocyte) resulting in a high surface area to volume ratio making the RBCs deformable. Central pallor can be observed to variable degrees in these species on examination of a peripheral blood smear. Central pallor is most prominent in the dog and less so in cats and horses. Poikilocytosis is a frequent finding in young ruminants and is considered to be normal, especially in goats (Fig. 23.1). In certain deer breeds, antelope and some breeds of sheep and goats, drepanocytes (also termed sickle cells) can be observed *in vitro*. This phenomenon occurs when the oxygen tension of blood is high (e.g. exposure to atmospheric oxygen during mixing of samples or smear preparation) or the pH is between 7.6 and 7.8. The sickling

is due to variants of hemoglobin that form insoluble, elongated polymers under these conditions.²²

The monolayer of the peripheral blood smear allows for evaluation of red blood cell arrangement. In most species, RBCs are individualized; however, some species are prone to rouleaux formation in which the RBCs line up in a column, resembling a stack of coins (Fig. 23.2). Although it may be a normal finding in cats and horses, it can be accentuated by elevated plasma protein concentrations.²² Rouleaux must be differentiated from agglutination. Agglutination is disorderly aggregation of RBCs (Fig. 23.3). Agglutination can be observed in immune-mediated hemolytic anemia (IMHA) and as an *in vitro* change in horses given heparin.²⁷ Rouleaux will be disrupted by diluting the blood with saline, but agglutination does not (see Chapter 140).

VARIATION IN ERYTHROCYTE MORPHOLOGY

Hemoglobin Content

Many species will have low numbers of circulating reticulocytes. This can result in noticeable polychromasia and anisocytosis. A polychromatophilic RBC contains organelles such as ribosomal RNA and

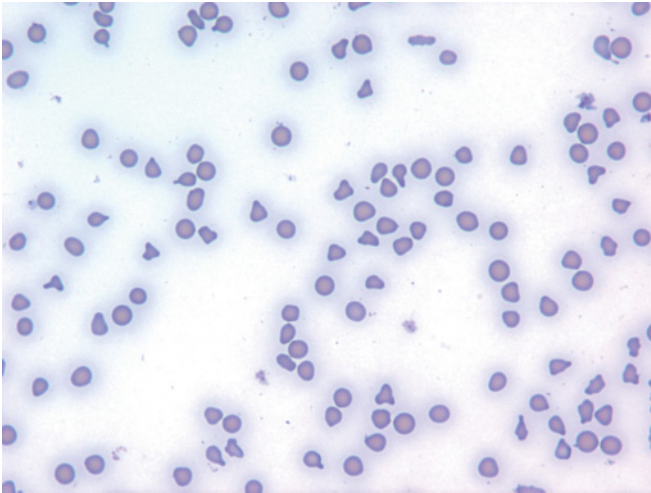


FIGURE 23.1 Blood smear from a goat showing typical poikilocytosis. Wright-Giemsa stain.

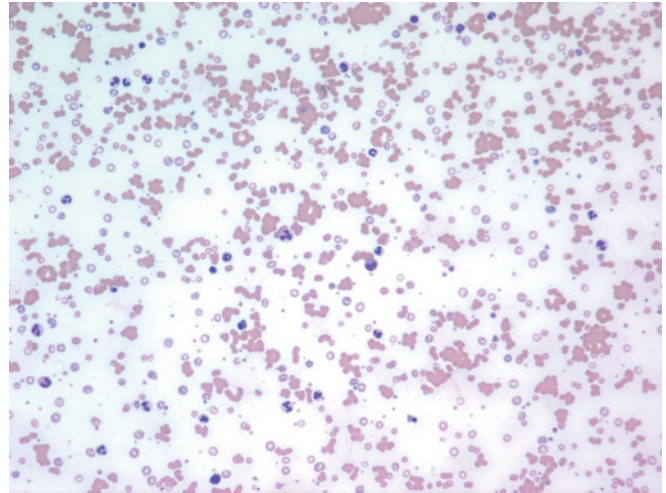


FIGURE 23.3 Blood smear from a dog with marked agglutination associated with immune-mediated hemolytic anemia. Wright-Giemsa stain.

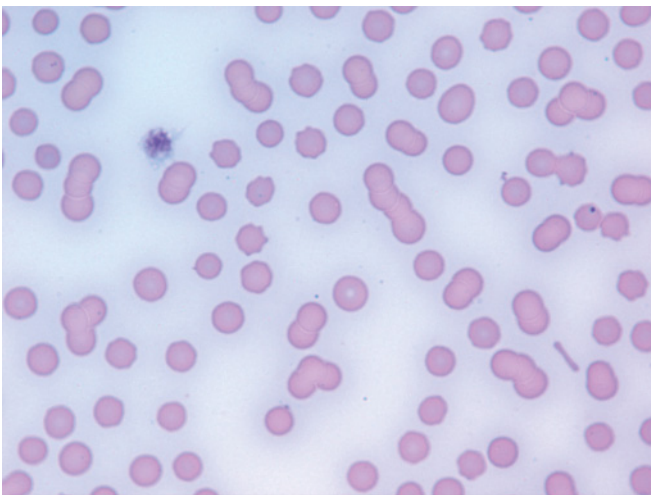


FIGURE 23.2 Blood smear from a cat showing rouleaux formation. Wright-Giemsa stain.

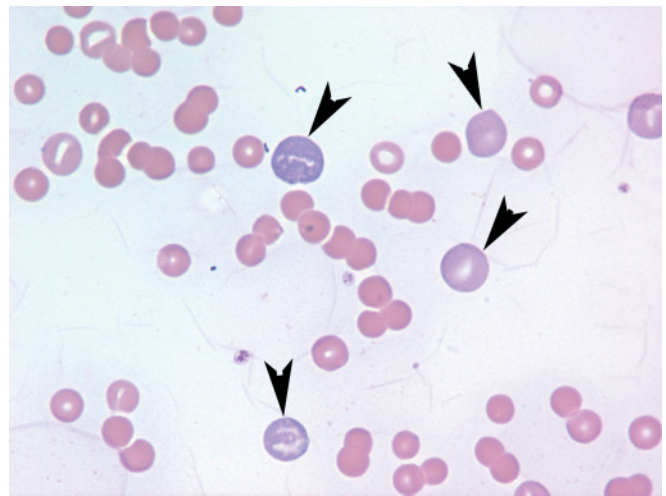


FIGURE 23.4 Blood smear from a dog showing polychromatophilic RBCs (arrows). These cells are larger than mature RBCs with grayish-blue cytoplasm. Wright-Giemsa stain.

mitochondria. When stained with a Romanowski stain, the presence of these structures will cause the cytoplasm to stain grayish-blue (Fig. 23.4). The RNA network and organelles precipitate, becoming more obvious if the blood is stained with new methylene blue (NMB). Increased polychromasia can indicate a regenerative response to anemia, but is not as sensitive as a reticulocyte count because not all reticulocytes are polychromatophilic. Horses do not have circulating reticulocytes and thus do not have polychromasia in their peripheral blood.

Patients with iron deficiency anemia can develop hypochromasia (see Chapter 26). Since iron is necessary for heme synthesis, erythrocytes from patients with iron deficiency can have decreased hemoglobin content. These cells have a thin rim of hemoglobin with increased area of central pallor (Fig. 23.5). True hypochromasia

needs to be differentiated from torocytes. A torocyte is usually considered an artifact.²² These cells have an increase in central pallor but it is an abrupt rather than a gradual decrease in the rim of hemoglobin. Other causes of hypochromasia include prolonged copper deficiency or deficiency of vitamin B6.¹⁷

Cell Size

Anisocytosis is a general term that describes variation in cell size. Healthy animals will have mild anisocytosis, likely from the presence of low numbers of circulating reticulocytes. Reticulocytes are often larger than mature erythrocytes so they are described as macrocytes. Other causes of macrocytosis include feline leukemia virus, myelodysplastic syndromes, and some congenital and hereditary conditions (see

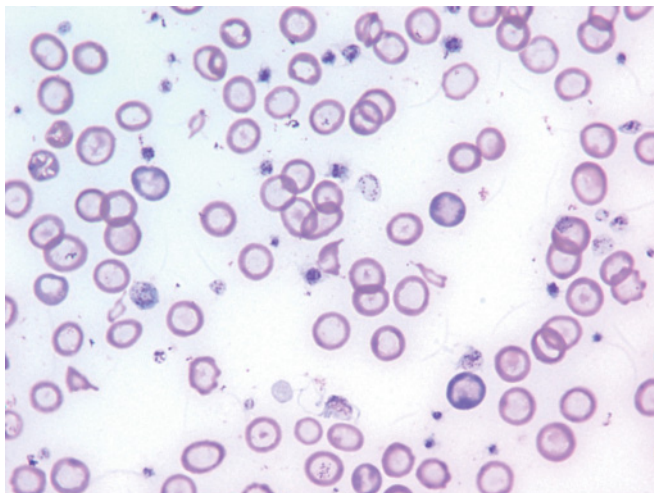


FIGURE 23.5 Blood smear from a dog with iron deficiency anemia. Notice marked hypochromasia. Wright-Giemsa stain.

Chapters 29, 30 and 66). Generally animals with these conditions will have an overall increase in their mean corpuscular volume (MCV). Microcytosis or small red blood cells can be observed with several different disease processes, the most common of which is iron deficiency (see Chapter 26). Due to the impaired heme synthesis, the red blood cells undergo an additional division. Other causes of microcytes include hereditary microcytosis (Akita and Shiba Inu) and familial dyserythropoiesis of English Springer Spaniels (see Chapter 30).^{15,22}

Poikilocytosis

Poikilocytosis is a general term for variation in RBC shape; it can occur in a variety of conditions, so poikilocytosis is non-specific. Further, some species are more prone to poikilocytosis than others. For instance, echinocytes are a common finding in pigs. Goat kids, less than 3 months of age and some adults, particularly adult angora goats are prone to marked poikilocytosis. Their RBC can vary in shape, and triangular, spindle, fusiform, matchstick, oblong and pear shaped erythrocytes can be observed. Anemic goats can form hemoglobin C which makes them more prone to poikilocytosis.^{22,24} Camelids, avians and reptiles have oval RBCs as their normal shape.

Specific changes occurring in the membrane structure, hemoglobin condition and bone marrow architecture can result in specific changes in RBC morphology. Acanthocytes have irregularly spaced spicules generally 2–20/cell. The name is derived from the Greek for “thorn” (Fig. 23.6). The changes in the RBC membrane of these cells are associated with increased cholesterol content of the membrane which is thought to expand the outer layer of the lipid bilayer; however, other studies suggest acanthocytes can also result from RBC fragmentation.⁴⁰ In dogs, liver disease, portosystemic shunts, hemangiosarcoma, disseminated intravascular

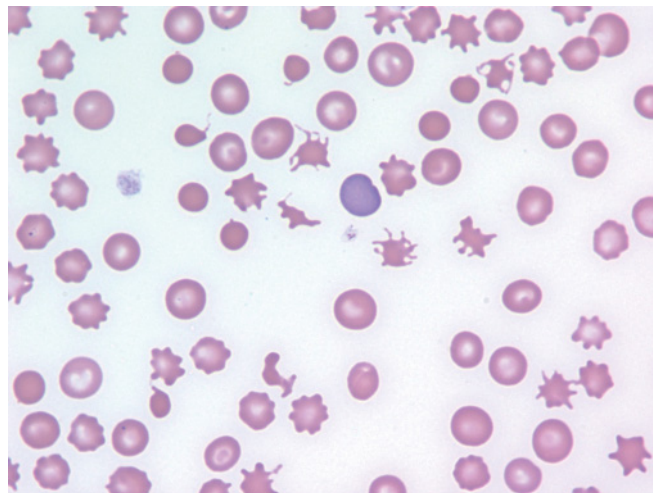


FIGURE 23.6 Blood smear from a dog showing acanthocytes with varying numbers of irregularly spaced projections. Wright-Giemsa stain.

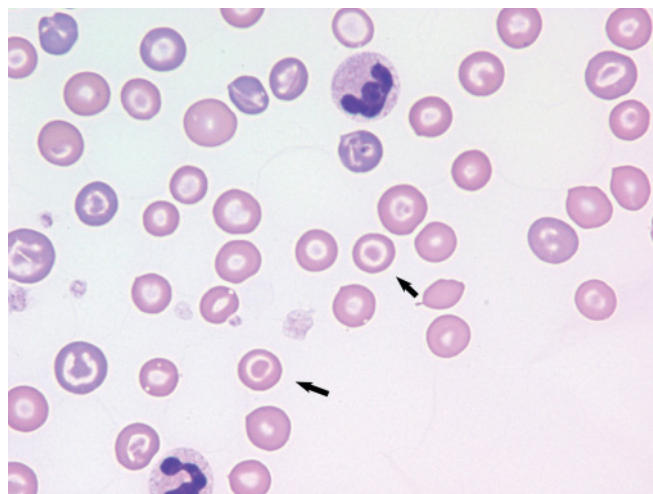


FIGURE 23.7 Blood smear from a dog with iron deficiency anemia. Codocytes are indicated with arrows. Wright-Giemsa stain.

coagulation, lymphosarcoma, glomerulonephritis, and high cholesterol diets have been associated acanthocytosis.^{19,40} In cats with liver disease, acanthocytes are reported as the most common poikilocyte.⁹

Codocytes

Codocytes (also termed target cells) resemble a bull’s eye (Fig. 23.7). They have a central area of hemoglobin surrounded by a pale rim with hemoglobin at the periphery of the cell. Similar to acanthocytes, codocytes have an increased amount of cholesterol resulting in an overall increase in the surface area of the erythrocyte membrane. Codocytes are often observed in patients with iron deficiency, cholestatic liver disease and post-splenectomy; however, codocytes are also frequent findings in regenerative anemias.

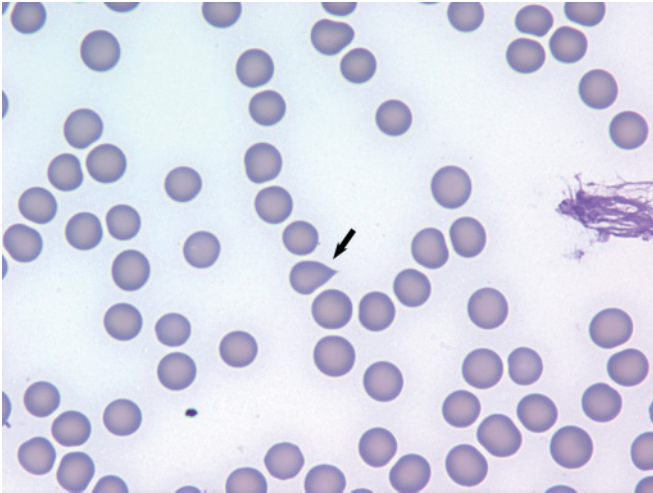


FIGURE 23.8 Blood smear from a dog. Dacryocyte is indicated with an arrow. Wright-Giemsa stain.

Dacryocytes

Dacryocytes are teardrop shaped cells that may occur when the RBC membrane is damaged and distorted as cells pass through narrow marrow or splenic sinusoids (Fig. 23.8).²² In human medicine, these cells are commonly associated with myelofibrosis or myelophthisis. In dogs they have been reported with myeloproliferative disorders and hypersplenism.

Eccentricocytes

Eccentricocytes (also termed erythrocyte hemighosts) are RBCs with eccentrically shifted hemoglobin within the cell, leaving a pale, hemoglobin-free area.¹⁸ These cells may progress to pyknocytes which are eccentricocytes in which the cell membrane has collapsed. Eccentricocytes have been reported to be easier to visualize on NMB stained preparations.³⁰ These cells are associated with oxidative damage to the erythrocyte membrane and cytoskeleton.⁶ In horses, eccentricocytes have been observed with red maple toxicity (*Acer rubrum*), erythrocyte glucose-6-phosphate dehydrogenase deficiency, and flavin adenine dinucleotide deficiency (see Chapter 36).^{18,35} In dogs, eccentricocytes have been observed with ingestion of substances known to cause oxidative damage, such as onions, vitamin K, garlic and acetaminophen. Additionally, eccentricocytes have recently been described in dogs with diabetes mellitus, T cell lymphoma and severe infections.⁶ Cattle receiving intravenous hydrogen peroxidase may have circulating eccentricocytes.³⁶

Echinocytes

Echinocytes are spiculated red blood cells with evenly dispersed, short projections. Their shape can vary from discoechinocytes to highly spiculated spherocytocytes or even ovaloecytocytes (Fig. 23.9). Echinocytes

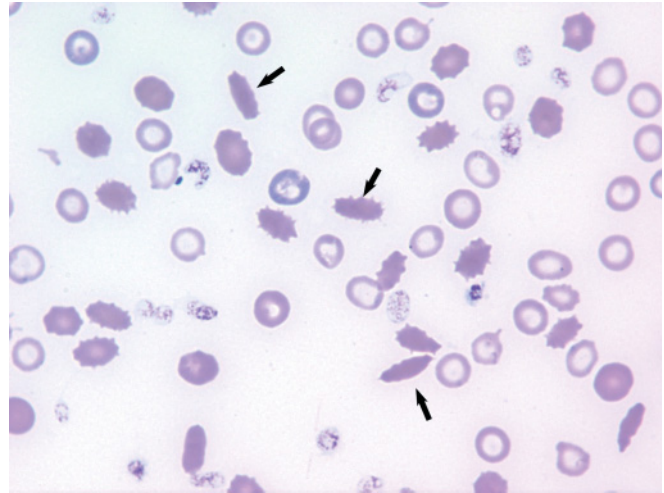


FIGURE 23.9 Blood smear from a dog. Ovaloecytocytes are indicated with arrows. Wright-Giemsa stain.

can be subclassified based on particular morphologic features. Type I echinocytes have irregular or angular edges but do not have distinct spicules. Type II echinocytes have multiple blunt spicules. Type III echinocytes are ovoid or spherical with 10–30 regularly spaced spicules. Echinocytes (also termed crenation) can occur as an artifact from sample handling, prolonged sample storage or during slide preparation. These cells occur commonly in pigs, *in vitro*.¹³ Echinocytes form when the outer leaflet of the lipid bilayer of the RBC membrane is expanded relative to the inner layer. Mechanisms of echinocyte formation include depletion of ATP, administration of amphipathic drugs, calcium loading, or when RBCs are dehydrated. In dogs, echinocytes have been reported with glomerulonephritis, lymphoma, hemangiosarcoma and other neoplasms, immune-mediated hemolytic anemia, pyruvate kinase deficiency, rattlesnake envenomation, and doxorubicin toxicosis among others.^{3,5} Cats likely have echinocytes with many of these diseases as well, but echinocytes have been specifically reported with chronic doxorubicin administration.²⁵ In horses, echinocytes have been observed with diseases causing hyponatremia and hypochloremia, such as colitis or exercise.^{14,22}

Elliptocytes

Elliptocytes are oval shaped, non-nucleated RBCs that are normal in camelid species. In people, hereditary ovalocytosis has been associated with a protein band 3 abnormality.³¹ In veterinary species, ovalocytes are occasionally described (Figs. 23.9 and 23.10). Hereditary ovalocytosis has been reported in a dog with protein band 4.1 deficiency,³⁴ but also in myelofibrosis, glomerulonephritis and myelodysplastic disease.²⁰ In cats, elliptocytes have been reported with myeloproliferative disorders, hepatic disease and with chronic doxorubicin administration.^{9,29}

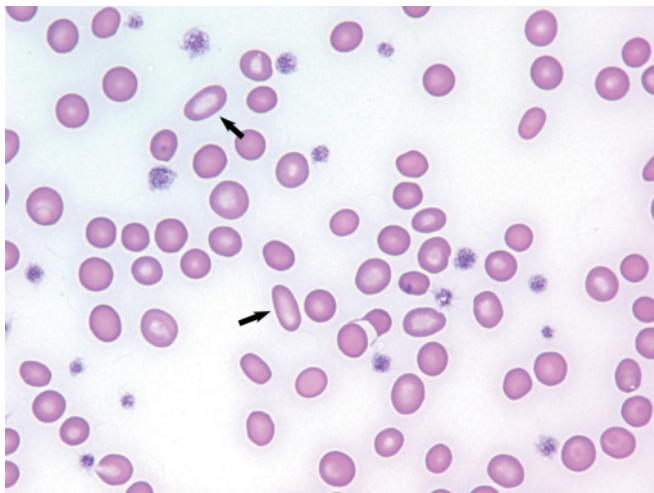


FIGURE 23.10 Blood smear from a dog. Ovalocytes are indicated with arrows. Wright-Giemsa stain.

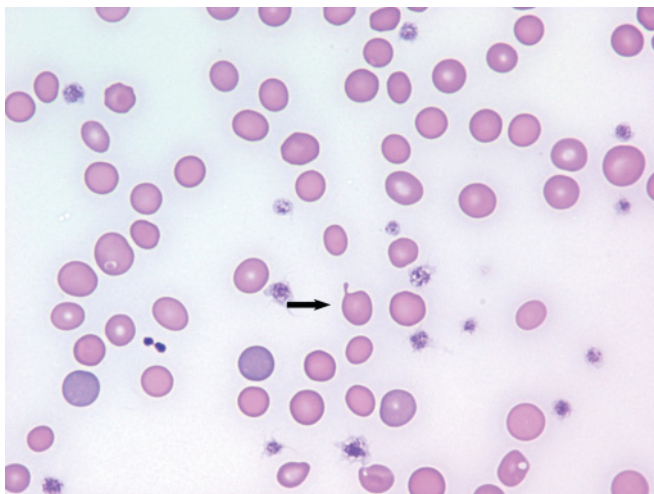


FIGURE 23.11 Blood smear from a dog. Occasional keratocytes are indicated by arrow. Wright-Giemsa stain.

Keratocytes

Keratocytes are spiculated RBCs that frequently have only 1–2 spicules and indicate RBC fragmentation (Fig. 23.11); thus, the RBC appears to have “horns” (kerat is the Latin term for horn). The formation of keratocytes has been associated with microvascular injury, especially when there is deposition of fibrin strands. The opposing sides of the damaged RBC may fuse leading to formation of a blister. Bursting of the blister is thought to result in progression to a keratocyte.²² Keratocytes have been reported in cats with liver disease⁹ and in dogs and cats with chronic doxorubicin administration and hemangiosarcoma.

Leptocytes

Leptocytes are thin RBCs that can fold and take on the shape of other poikilocytes such as codocytes, knizo-

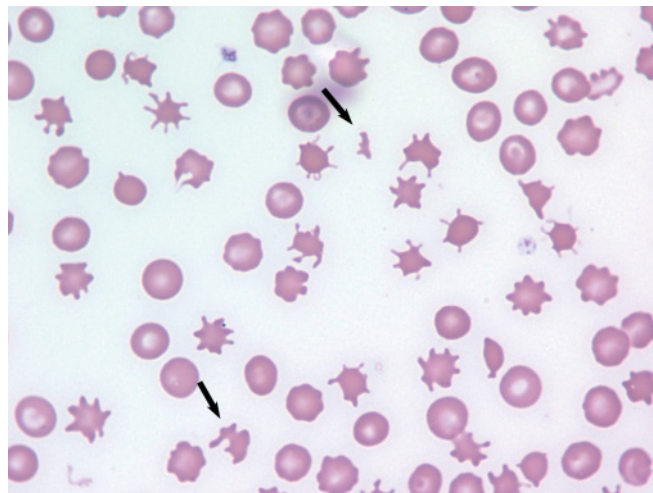


FIGURE 23.12 Blood smear from a dog. Severe poikilocytosis with schistocytes indicated with arrows. Wright-Giemsa stain.

cytes (central fold giving the appearance of a central bar of hemoglobin) or stomatocytes. Leptocytes can be observed in iron deficiency and cholestatic liver disease.²²

Schistocytes

Schistocytes are red blood cell fragments usually resulting from direct physical damage to RBCs secondary to vascular abnormalities or turbulent blood flow. The shape of the fragments may vary from pointed or triangular to spiculated (Fig. 23.12). Microangiopathic fragmentation has been described in dogs in a several different disorders including disseminated intravascular coagulation (DIC), glomerulonephritis (GN), hemangiosarcoma, myelofibrosis, dyserythropoiesis, and chronic doxorubicin toxicosis.^{3,11,40} Schistocytes have also been identified as a common feature of DIC in calves.^{34,21} However, they are uncommon in the horse and cat,¹² though schistocytes have been reported in cats with hepatic disease.⁹

Spherocytes

Another result of RBC fragmentation is spherocytes. Spherocytes are RBCs that have lost their biconcave shape and have become round like a sphere. On a blood smear, these cells have lost their central pallor and appear slightly darker and often a little smaller than healthy RBCs (Fig. 23.13). IMHA is a common cause of this poikilocyte. They occur secondary to antibody binding on the membrane or complement fixation. Part of the membrane may be phagocytized or just punctured resulting in the change in shape. These cells have been associated with other diseases as well, including coral snake, rattlesnake and bee sting envenomation, and anaplasmosis infection in cattle.²⁸ Spherocytosis have been reported in Basenji's with pyruvate kinase deficiency and in goat erythrocytes treated with lysolecithin.^{5,22,23} Spherocytes are most obvious

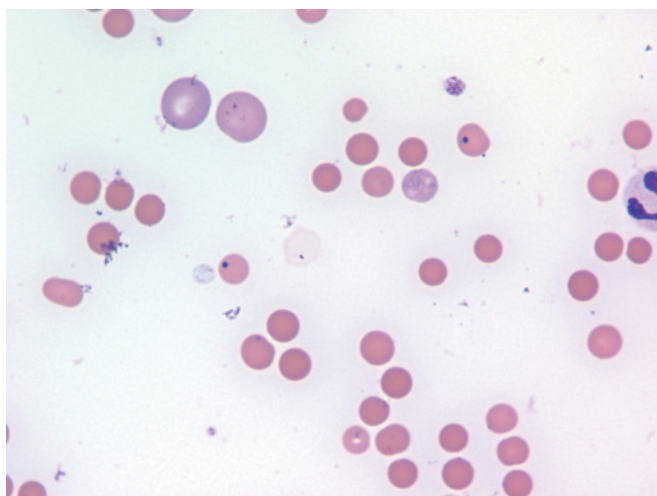


FIGURE 23.13 Blood smear from a dog showing many spherocytes. Wright-Giemsa stain.

in dogs, likely because they have prominent central pallor. In other species such as the cat and horse, spherocytes are difficult to identify.

Not all spherocytes are formed secondary to fragmentation. Spherocytes can be caused by a molecular defect in one or more of the proteins of the RBC cytoskeletal network. Double knock-out mice with defects in actin binding proteins affect erythrocyte shape and membrane integrity leading to pronounced spherocyte formation.⁸ Spectrin deficiency has been identified in a group of Dutch Golden Retrievers (see Chapter 29).³³

Stomatocytes

Stomatocytes are RBCs that have a narrow elongated band of central pallor resembling a mouth on dried blood smears. In actuality, these cells are more bowl-shaped in wet-mount preparations. Several breeds of dogs have been identified with hereditary stomatocytosis, including Alaskan Malamutes with concurrent chondrodysplastic disease, Drentse patrijshond with stomatocytosis-hypertrophic gastritis and Miniature and Standard Schnauzers (see Chapter 29).^{4,32} The Drentse patrijshonds often have a normal MCV whereas the Schnauzers and Malamutes have an increased MCV.^{4,32}

Hemoglobin Crystals

Hemoglobin crystals are unstable hemoglobin that precipitates in the RBC. These dense staining, rhomboid or rod shaped crystals lead to decreased deformability of the cell (Fig. 23.14). Crystals may be found in human patients with Hemoglobin C disease and sickle cell anemia, particularly after splenectomy. In veterinary medicine, hemoglobin crystals are infrequently reported and their cause is uncertain. Nonetheless, they have been described in dogs, llamas and horses with FAD deficiency.^{7,35}

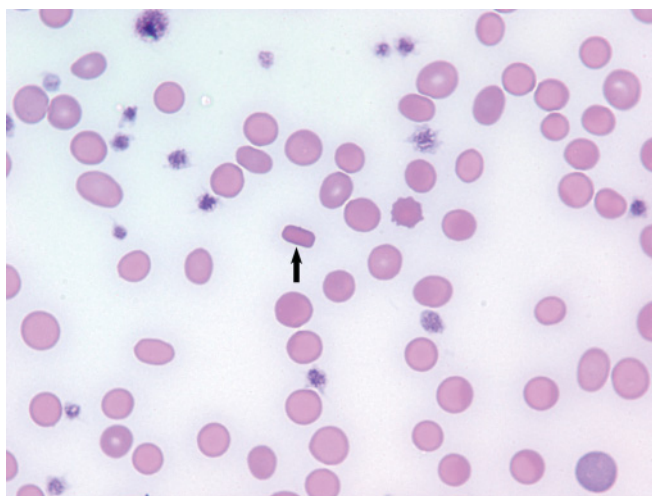


FIGURE 23.14 Blood smear from a dog showing hemoglobin crystals (arrow). Wright-Giemsa stain.

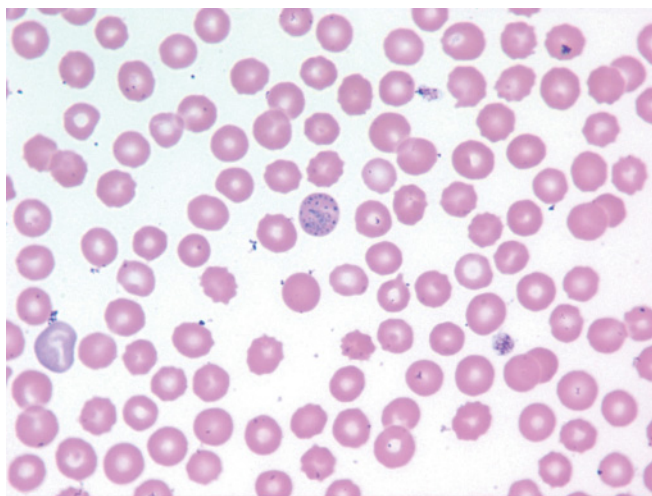


FIGURE 23.15 Blood smear from a dog showing basophilic stippling. Wright-Giemsa stain.

Erythrocyte Inclusions

Basophilic stippling represents the spontaneous aggregation of ribosomes and polyribosomes in RBC. In Romanowsky-stained samples, affected RBCs contain uniformly distributed punctate, basophilic structures (Fig. 23.15). Basophilic stippling may be seen in cattle and other ruminants as a part of the regenerative response to anemia. It may be associated with exuberant regeneration in other species including the dog and cat. However, if basophilic stippling and increased numbers of nucleated RBC (nRBC) are present in the absence of a severe anemia, lead toxicity should be considered.

Nucleated RBCs in circulation can also be part of a regenerative response to anemia. These cells, depending on their level of maturity, usually have polychromatophilic or hemoglobinized cytoplasm and a round

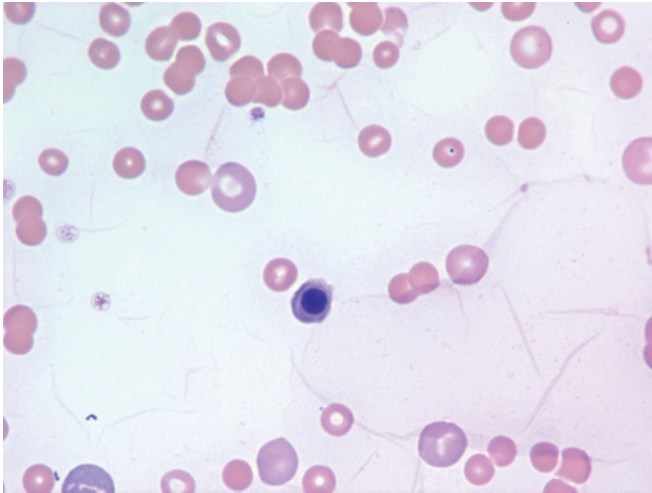


FIGURE 23.16 Blood smear from a dog showing occasional metarubricytes as part of a regenerative response. Wright-Giemsa stain.

nucleus (Fig. 23.16). The metarubricytes have a small, pyknotic nucleus but less mature cells have a stippled chromatin pattern. When nRBCs accompany a regenerative response, polychromasia and anemia should also be observed. However nRBCs may occur in the absence of anemia in diseases affecting the spleen and bone marrow. Myeloproliferative disorders and infiltrative marrow diseases such as solid tumor metastases and myelofibrosis can result in high numbers of nRBCs, occasionally with less mature erythrocyte precursors, rubriblasts, and prorubricytes in circulation. Low numbers of circulating nRBCs can be observed in splenectomized animals or in dogs or cats with hemangiosarcoma, hemophagocytic histiocytic sarcoma, and pure red cell aplasia.^{16,39} Cats with myelodysplastic syndromes, hepatic lipidosis, upper respiratory infections and hemangiosarcoma can have normoblastemia.¹⁶ Lead toxicity is commonly associated with metarubricytosis; however, these patients are usually not anemic.

Nuclear remnants, not expelled as RBCs leave the bone marrow, are termed Howell-Jolly (HJ) bodies. They are round, deeply basophilic structures that may vary in size (Fig. 23.17). Howell-Jolly bodies are normally removed by the spleen. However, in cats and horses, species having non-sinusoidal spleen, low numbers are normally seen in circulation. Increased numbers of HJ bodies can be seen as part of a regenerative response to anemia, in animals with hypofunctioning spleens, or in splenectomized patients. There also are reports of increased numbers of HJ bodies in non-anemic miniature and toy poodles with hereditary macrocytosis (see Chapter 30).

Oxidative damage can result in a number of morphologic changes to the RBCs including the formation of Heinz bodies.^{1,2,10,26,41} Heinz bodies are small aggregates of oxidized hemoglobin that can occur as hemoglobinized projections extending from the cell or as pale areas within the cell (Fig. 23.18A). With Romanowsky stains, the Heinz bodies are pale or hemoglobinized but

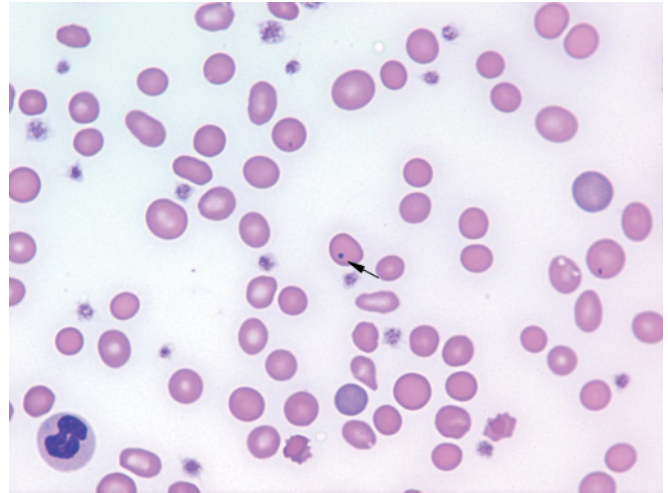


FIGURE 23.17 Blood smear from a dog. Howell-Jolly bodies are nuclear remnants observed in the cytoplasm of red blood cells (arrow). Wright-Giemsa stain.

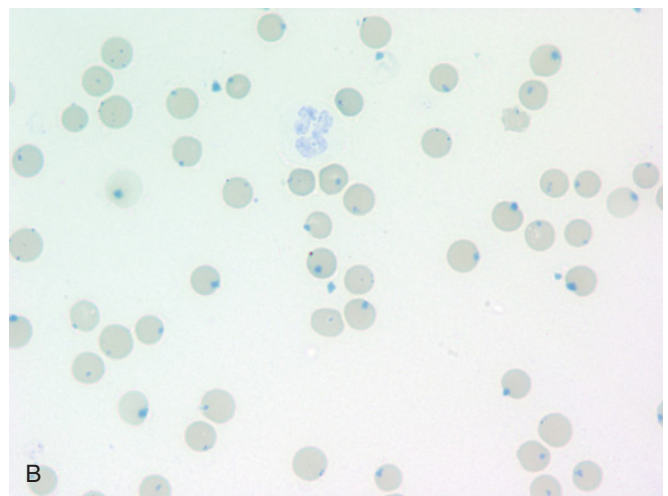
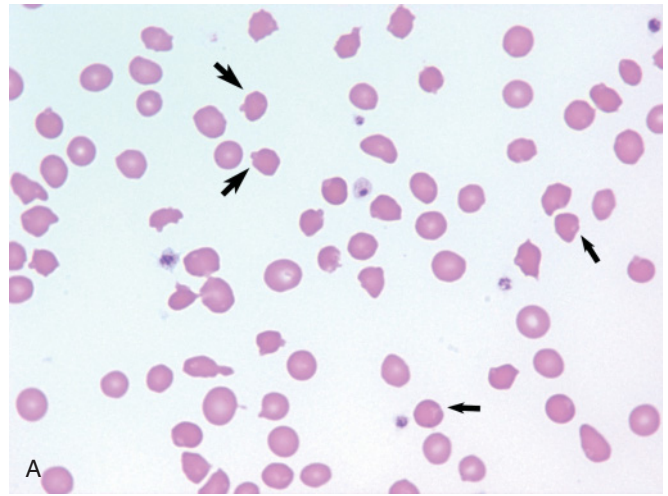


FIGURE 23.18 Blood smear from a cat. (A) Wright-Giemsa stain: Many Heinz bodies are observed and can appear as projections from the RBC membrane (large arrow) or as pale areas near the membrane (small arrow). (B) New methylene blue stain: Heinz bodies are more obvious with vital stains.

with NMB, they are bluish-green and much more obvious (Fig. 23.18B). Many toxins and diseases have been reported to cause Heinz bodies (see Chapter 36).

Iron observed in mature RBCs is referred to as siderocytes or Pappenheimer bodies. With Romanowsky stain, the iron appears as irregularly shaped, pale basophilic material, whereas ferric iron forms a bright blue pigment when stained with Prussian blue. Siderocytes have been reported in the blood in association with myeloproliferative disorders, lead toxicity, and hemolytic anemia. Sideroblasts are nucleated erythroid precursors with iron in their cytoplasm. These cells have been reported with myelodysplastic syndromes and myeloproliferative disorders and in inflammatory diseases in dogs and cats.^{37,38}

Artifactual changes can occur that mimic true pathologic fragmentation or inclusions. Stain precipitate or drying artifact can mimic red blood cell parasites or inclusions such as HJ bodies. A commonly observed artifactual change associated with prolonged drying of blood smear preparations, produces refractile RBC inclusions. Additionally, crenation of RBCs can occur as an artifact of drying. Usually artifactual crenation will occur in thick areas of blood smear.

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Laboratory and Clinical Diagnosis of Anemia

HAROLD TVEDTEN

Clinical Manifestations of Anemia
 Presence and Severity of Anemia
 Classification Systems
 Classification by Strength of Erythropoiesis
 Classification by Erythrocyte Volume and Hemoglobin Concentration

Classification by Blood Smear Morphology
 Classification by Etiology
 Diagnostic Approaches
 Blood Loss Anemia
 Non-Regenerative Anemia
 Bone Marrow Evaluation

Acronyms and Abbreviations

Advia, Siemens Advia 2120® hematology system; CBC, complete blood count; CRP, corrected reticulocyte percentage; EDTA, ethylenediaminetetraacetic acid; EIA, equine infectious anemia; FeLV, feline leukemia virus; fL, femtoliter; Hct, hematocrit; Hgb, hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; NRBC, nucleated red blood cell; PCV, packed cell volume; PRCA, pure red cell aplasia; RBC, red blood cell; RDW, red cell distribution width; RPI, reticulocyte production index; Sysmex, Sysmex XT 2000iV® hematology system; WBC, white blood cell.

This chapter discusses diagnosis of anemia based primarily on laboratory findings. Erythrocyte morphology (see Chapter 23) and specific causes of anemia are described elsewhere, except for blood loss anemia which will receive more attention in this chapter. Diagnosis of the cause of anemia uses certain characteristics of blood to place the anemia into pre-established classes or categories useful for diagnosis. Several classification systems are used and are based on various tests and observations. The systems overlap and classifications by one system (e.g. macrocytic hypochromic anemia) may be identifying the same change or process in red blood cells (RBCs) as another classification (e.g. reticulocytosis and regenerative anemia). Thus different names frequently mean the same in terms of diagnosis. Despite the classification system used, the goal of diagnosis should be to provide the most specific and correct diagnosis to allow appropriate treatment or other action by the attending clinician.

CLINICAL MANIFESTATIONS OF ANEMIA

The clinical signs that prompt an animal's owner to seek a clinician to diagnose and then treat an anemia include weakness, lack of stamina, pale mucous membranes,

icterus and hemoglobinuria (red urine). The purpose of laboratory testing is to provide unbiased evidence that supports or refutes a clinical or tentative diagnosis. Laboratory diagnosis of the cause of anemia should be based first on laboratory conclusions founded on test results and interpretation of cell morphology in blood smears and graphics from hematology instruments. Only after making those conclusions should clinical signs, age, breed, and other clinical information be considered and allowed to influence the conclusions or diagnosis. This permits laboratory results to indicate new possibilities and avoid "premature closure" too early in diagnosis. Additional, more specific testing may then be needed.

PRESENCE AND SEVERITY OF ANEMIA

The first step in evaluation is to determine if the animal has anemia and, if present, to evaluate severity (Table 24.1). Laboratory parameters routinely used include hematocrit (Hct), total erythrocyte count (RBC count), and hemoglobin (Hgb) concentration. Packed cell volume (PCV) is essentially the same as Hct, though implies a centrifugal method of analysis. These values (Hct, RBC count or Hgb concentration) should be simi-

TABLE 24.1 Guidelines for Classification of Severity of Anemia

	Dog	Horse	Cat/Ruminant
Mild	30–37 ^a	30–33	20–26
Moderate	20–29	20–29	14–19
Severe	13–19	13–19	10–13
Very Severe	<13	<13	<10

^aValues are hematocrit or packed cell volume in percent.

larly reduced in proportion to the reduction of erythroid mass and should similarly reflect the severity of anemia in the animal. Hematocrit will be used in this chapter to describe severity of anemia but Hgb concentration is used more frequently in Europe and also in human medicine.

If Hct, RBC count, and Hgb concentration are not similarly decreased in anemia, then erythrocytes are not normal in size and Hgb content. Changes in erythrocyte indices (MCV, MCHC) should help explain any discordance in relative decreases (see section on classification by erythrocyte volume and Hgb concentration later in this chapter). For example, in iron deficiency anemia with severe microcytosis, RBC count will not be decreased as much as Hgb concentration or Hct and, therefore not reflect the severity of the anemia as well. Even normal numbers of microcytic erythrocytes will not contribute to a normal Hgb concentration per unit volume of blood and the smaller erythrocytes will be packed into a smaller volume of the blood (PCV).

Pre-analytical and analytical errors may also cause discordance among Hct, Hgb concentration, and RBC count. For example, Heinz bodies may make erythrocytes more fragile so they lyse in the microhematocrit centrifuge, lowering the PCV. Heinz bodies remaining in suspension after erythrocytes are lysed, increase the optical density and falsely increase Hgb concentration. Heinz bodies in erythrocytes make them more optically dense to laser hematology instruments also causing a falsely increased cell Hgb concentration and MCHC.

Severity of an anemia is useful in diagnosis. Table 24.1 provides some guidelines for classifying severity. Moderate to severe anemias are more likely important or primary problems. For example, primary bone marrow disorders like chronic myelofibrosis can cause very severe anemia (e.g. Hct 6–8%) while secondary suppression of bone marrow (e.g. anemia of inflammation) is characteristically mild to moderate. Severe anemia or rapid decline in erythroid mass should stimulate more aggressive diagnosis and treatment, while mild to moderate anemia in animals with severe inflammatory or neoplastic disorders is expected as secondary problems.

Clinical information such as breed and age affect interpretation (see Chapter 131). Sight hounds like greyhounds normally have higher Hct (about 50–65%) compared to other dog breeds (37–55%) and, therefore a mild anemia in a greyhound will have a Hct within the reference intervals for dogs in general. Puppies have

TABLE 24.2 Canine and Feline Relative and Absolute Reticulocyte Guidelines

Strength of Regeneration	Canine Reticulocytes (%)	Feline	
		Aggregate Reticulocytes (%)	Punctate Reticulocytes (%)
Relative			
None	1 ^a	0–0.4 ^a	1–10 ^a
Weak	1–4	0.5–2	10–20
Moderate	5–20	3–4	20–50
Strong	21–50	>5	>50
Absolute			
None	60 ^b	<15 ^b	<200 ^b
Weak	150	50	500
Moderate	300	100	1000
Strong	>500	>200	>1500

^aValues are percentage of non-nucleated RBCs that are reticulocytes. The percentage of canine reticulocytes or feline aggregate reticulocytes also may be used to convert the percentage of polychromatophils on a blood smear to strength of polychromasia and thus erythropoiesis.

^bReticulocytes × 10⁹/L or reticulocytes × 10³/μL.

lower Hct than adult dogs (as well as lower plasma protein and higher reticulocyte numbers). For example a 6-week-old puppy can have a Hct of 26–30%, reticulocytes of 4.5% and plasma protein of 5.0–5.6 g/dL. Thus a normal puppy can appear to have a regenerative, blood loss-type anemia when using adult reference intervals.

Hydration status must be considered. Hematocrit reflects severity of anemia only when the animal has normal hydration and blood volume. It may take 1–2 days after blood loss before blood volume is restored to normal and the Hct shows the severity of the anemia.² Even acute hemolytic anemia may not have a Hct reflecting the severity of anemia at the time of presentation to a clinic.

CLASSIFICATION SYSTEMS

Classification by Strength of Erythropoiesis

Evaluation of bone marrow function divides anemia into those with variably reduced or ineffective erythropoiesis (non-regenerative) or with active and effective erythropoiesis (regenerative) (Table 24.2). Anemia not caused by primary or secondary bone marrow dysfunction should have appropriate evidence of erythropoiesis (regeneration or responsive). The anemia is classified as regenerative if signs of increased erythropoiesis seem adequate for the severity of anemia, duration, species characteristics, treatments, and likelihood of multiple etiologies.

Regenerative anemias are caused by loss of erythrocytes from the body (external blood loss) or lysis of the erythrocytes within the body (hemolytic anemia and internal blood loss). These are easier to diagnose than the non-regenerative anemias.

Bone marrow regeneration in species that display consistent reticulocyte responses (e.g. dog, cat, pig, and rat) is primarily evaluated by the reticulocyte response (see chapters on individual species characteristics). The strength of increase in the absolute reticulocyte count per unit volume of blood best reflects how effective bone marrow erythropoiesis is in the patient (Table 24.2). The percentage of reticulocytes is a relative value affected by the severity of anemia and number of mature erythrocytes remaining. Absolute counts ($\% \text{ reticulocytes} \times \text{erythrocytes}$) are preferred for interpretation. The corrected reticulocyte percentage (CRP) and reticulocyte production index (RPI) are other methods for interpreting the reticulocyte response when the total erythrocyte count is not available to calculate an absolute reticulocyte count. Guidelines for and the clinical usefulness of CRP or RPI are not well established in veterinary species.

Reticulocyte numbers are best interpreted at the time of an expected peak of reticulocytes at 4–8 days after onset (see Fig 24.5A). Guidelines for interpretation of reticulocyte numbers are for the peak reticulocyte

response and may be misleading early in an anemia (i.e. pre-regenerative) when reticulocyte numbers have not increased yet, or late in an anemia when reticulocyte numbers decline (10–14 days).⁶ Late in an anemia the number of macrocytic hypochromic erythrocytes (Fig. 24.1) is more reflective of the strength of primary regenerative response than the reticulocyte number, which may be declining or back to normal.

Feline punctate reticulocytes have a long half-life in blood and therefore may accumulate in large numbers (Table 24.2). The kinetics of feline aggregate reticulocyte responses are similar to those of other species but maximal responses are weaker (Table 24.2) than expected in dogs.⁶ Aggregate reticulocytes may not be released unless the anemia is severe.

Microscopic identification of aggregate and punctate reticulocytes is time consuming and subjective when many reticulocytes are present (see Chapter 136). The automated reticulocyte counts of the Advia 2120® and Sysmex XT 2000® detect primarily feline aggregate reticulocytes and not punctate reticulocytes.³ A time saving approach is to use automated feline reticulocyte

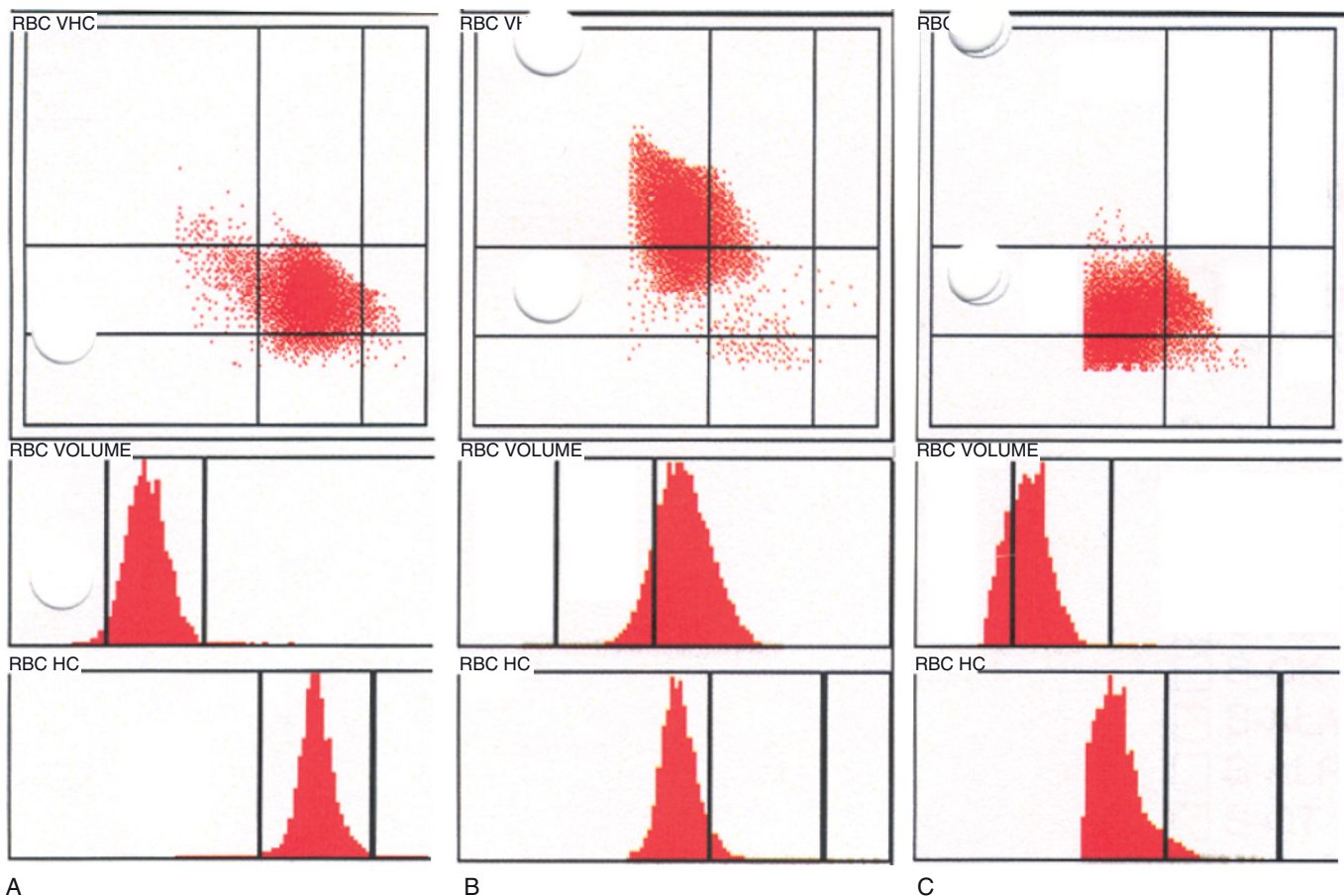


FIGURE 24.1 Advia 2120 RBC cytograms, RBC volume histograms and Hgb concentration histograms from three dogs illustrating the three main types of anemia based on size and hemoglobin concentration of RBCs. (A) The first dog had non-regenerative IMHA and had no reticulocytes or macrocytic cells. It was a normocytic normochromic anemia with almost all RBCs in the central box of the 9-box RBC cytogram. (B) This dog had phosphofructokinase deficiency and hemolytic anemia. Almost all RBCs were macrocytic hypochromic due to a marked regenerative anemia. Few RBCs remained in the central box of the 9-box RBC cytogram. (C) Dog with iron deficiency and microcytic hypochromic anemia. Its erythrocytes were more hypochromic than microcytic. Its reticulocytes were also microcytic.

counts for accurate aggregate reticulocyte counts and a quick screening of a new methylene blue-stained blood smear for an estimate of punctate reticulocyte percentages.

It is important that laboratories indicate the type of feline reticulocyte reported. Sometimes only “reticulocytes” are reported without specifying the type. Note in Table 24.2, that the number of aggregate reticulocytes indicating a strongly regenerative anemia is the same number as the number of punctate reticulocytes indicating no regeneration. Duration of the feline regenerative response may be interpreted by pattern of changes in the two types of reticulocytes. A strong increase in aggregate reticulocytes but mild increase in punctate reticulocytes indicates an early days response (3–6 days after onset of anemia), while late in a response (9–20 days) there may be many punctate reticulocytes but no increase in aggregate reticulocytes.⁶

Other indicators of an appropriately active bone marrow in anemia are less specific for erythropoiesis than reticulocytosis; however, these may be useful in species without consistent reticulocyte responses. Nucleated RBCs (NRBCs) are useful indicators in ruminants (e.g. cattle, llamas) to reflect regeneration.⁵ NRBC numbers are frequently reported as a relative ratio of NRBC/100WBC, so marked changes in WBC count can affect this ratio. Basophilic stippling, especially in ruminants, and Howell-Jolly bodies suggest a regenerative anemia. RBCs produced during increased erythropoiesis are larger; thus macrocytosis and anisocytosis suggest active erythropoiesis and may be the only indicators in peripheral blood of horses that release very few reticulocytes into circulation. Macrocytosis and anisocytosis can be detected by the MCV (e.g. >52 fL in horses), RBC cytograms and histograms (Fig. 24.2) or blood smear evaluation. RBC distribution width (RDW) indicates the amount of anisocytosis. NRBC, macrocytosis, Howell-Jolly bodies and anisocytosis are caused by factors other than regenerative anemia such as glucocorticoid treatment, heat stroke, dysmyelopoiesis, myeloproliferative disorders, prolonged storage of EDTA blood etc., and are thus not specific indicators of regeneration.

Classification by Erythrocyte Volume and Hemoglobin Concentration

Erythrocyte volume changes are microcytic (smaller), normocytic or macrocytic (larger). Erythrocyte hemoglobin concentration changes are hypochromic (reduced) and normochromic (normal concentration). Hyperchromic changes indicate an erroneous result and not a disease-related change. Pre-analytic and analytic errors, such as hemolysis or Heinz bodies, cause artifacts that imply the erythrocytes contain more hemoglobin per unit volume than a normal cell filled with hemoglobin.

The three important diagnostic patterns are macrocytic hypochromic anemias (regenerative anemias with large, young RBCs that are not fully hemoglobinized), normocytic normochromic anemia (non-regenerative

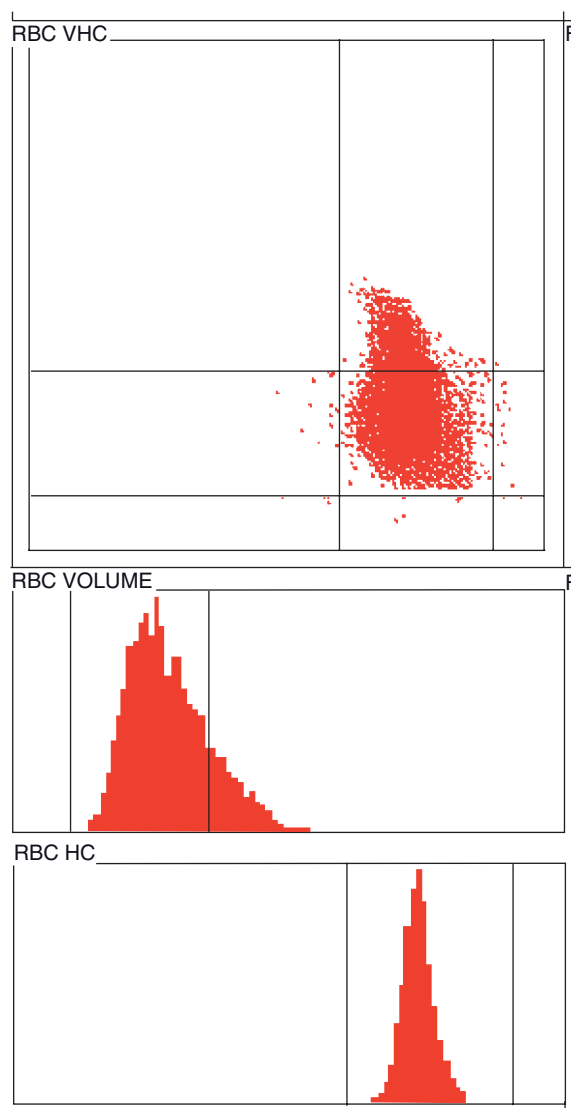


FIGURE 24.2 Regenerative response in a horse with immune-mediated hemolytic anemia. There is a macrocytic normochromic population of RBCs seen in the Advia 2120 RBC graphics. This horse did not have reticulocytes detected by the Advia 2120.

anemias with residual normal erythrocytes), and microcytic hypochromic anemias, that are usually iron deficiency anemias.

The MCV and MCHC have been used for this classification. MCV and MCHC are unfortunately too insensitive and frequently fail to correctly differentiate the three main patterns. The numbers of abnormal erythrocytes are usually small and values for MCV or MCHC frequently remain within reference intervals.

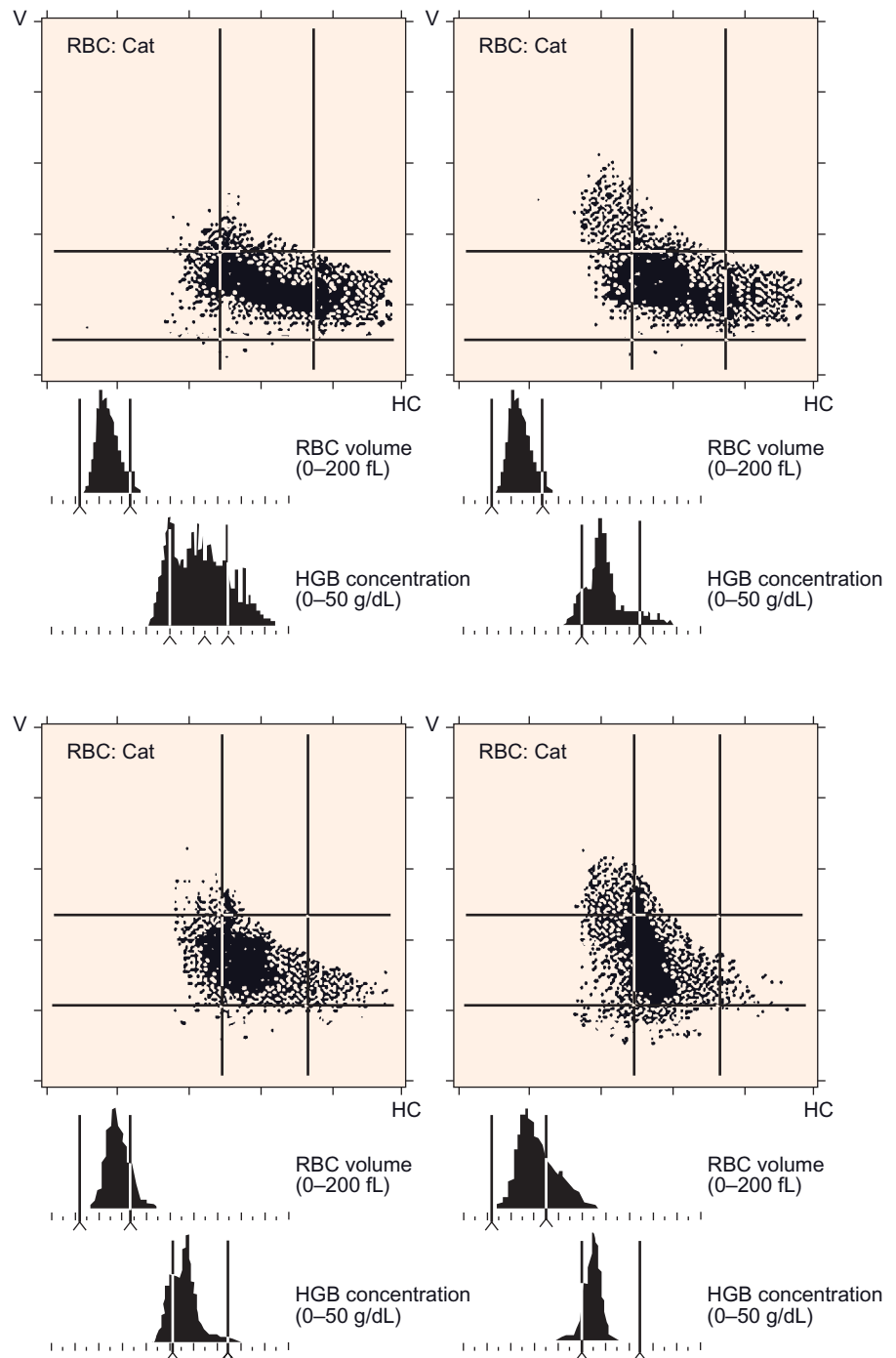
Computer graphics of the Advia 2120® are more sensitive and specific in detecting diagnostic changes in volume or Hgb concentration of RBCs than the MCV or MCHC (Fig. 24.1), especially with small populations of abnormal RBCs. Advia’s two dimensional RBC cytogram displays and records each RBC by individual cell volume and cell hemoglobin concentration. Advia’s volume and Hgb concentration histogram show well

the relative size of populations of erythrocytes. Percentages of macrocytic, microcytic, or hypochromic RBCs or reticulocytes are also available from the Advia (unfortunately the somewhat round groups of different types of RBCs are not confined to the square shaped, nine counting boxes in the RBC cytograms and these percentages still must be subjectively interpreted while looking at the histograms; Fig. 24.1). However, these are exceptionally good tools in anemia diagnosis. For example, microcytic hypochromic cells can be seen in iron deficiency when the MCV and MCHC are normal.

In horses, that have only minimal reticulocyte responses, the Advia cytograms and histograms still demonstrate macrocytosis in strongly regenerative anemias (Fig. 24.2). Cats have relatively weak aggregate reticulocyte responses, which often fail to correctly affect MCHC and MCV, but may be noted in Advia's RBC graphics (Fig. 24.3).

Macrocytic normochromic anemia is the normal regenerative response of horses (Fig. 24.2). In cats macrocytic normochromic erythrocytes in the absence of polychromasia suggests feline leukemia virus (FeLV)

FIGURE 24.3 Composite of four sets of RBC cytograms and histograms over 1 week from a cat with Heinz body anemia from eating baby food containing onion powder. The third set (lower left) looks the most normal and may be used for comparison. The RBC cytogram has one dark, round cluster that is the normocytic normochromic erythrocytes. The cells should be in the center box but the grid was not aligned well. The lightly dispersed dots extending to the right are RBCs with Heinz bodies that appeared hyperchromic. Note on the first set (upper left) this extension is very dark indicating many RBCs appeared hyperchromic because 84% contained large Heinz bodies. The MCHC was falsely high (44g/dL) on that day. The Hgb concentration histogram was bimodal. The smaller left peak was normochromic cells (grid was offset) and the large, wider right peak was the hyperchromic appearing cells. The final set of graphics (lower right) shows a good regenerative response with mainly macrocytic and slightly hypochromic RBCs extending up and to the left of the normal cells. The RBC volume histogram shows many of the cells were macrocytic. The HGB histogram shows essentially no hyperchromic cells (RBCs with Heinz bodies dropped to 17%) and a small population of hypochromic cells (i.e. reticulocytes). The sequence shows well the loss of hyperchromic erythrocytes with Heinz bodies and the developing regenerative response with young, macrocytic cells.



infection or myelodysplastic syndromes. Some poodles have macrocytic erythrocytes as a familial defect. Swelling of erythrocytes is a common artifact in day old samples. Altered erythrocytes may also swell as in stomatocytosis in dogs. Vitamin B12/folic acid deficiency is a rare condition in domestic animals. Additionally, when it occurs, it is not a cause of macrocytic normochromic anemias. Cobalamin (vitamin B12) malabsorption has been reported in Giant Schnauzers but they had a normocytic normochromic anemia (see Chapter 30).¹

Classification by Blood Smear Morphology

Blood smear evaluation is an essential procedure in classification of anemia, especially hemolytic anemias. Many observations critical to diagnosis can only be made or are made more accurately by a trained microscopist. Some examples include identification of blood parasites, spherocytes, autoagglutination, Heinz bodies, hypochromasia, eccentrocytes, pyknocytes, erythrocyte fragmentation (Table 24.3), brown color of methemoglobin, and clumping of platelets or leukocytes (see Chapter 23).

Classification by Etiology

The ultimate goal of diagnosis is correct classification of anemia by etiology or etiologies in case of multiple causes (see chapters on specific causes and Tables 24.3–24.5). The path to the final diagnosis varies with each case. The likely etiology may be identified in the history. Substances known to cause Heinz body anemia, such as acetaminophen/paracetamol in cats or red maple tree branches and leaves in horses, can be identified by proper questioning of the owner.

DIAGNOSTIC APPROACHES

Anemia diagnosis usually begins with a complete blood count (CBC) including blood smear evaluation and, in many species, reticulocyte evaluation. If the diagnosis is not obvious from the history, physical examination, and CBC, then additional testing is indicated. Additional tests are chosen based on the situation, species, and probability of different causes of anemia for a location. Examples include hemoglobinuria in a young dog with a tendency to eat foreign bodies, which should indicate that radiographs are taken to look for zinc-containing metallic foreign bodies in the stomach.

A simple, algorithmic approach to selected anemias reflects the previous discussion (Fig. 24.4). One must add one's own interpretation and knowledge to any algorithm to consider additional information or factors in a given situation. After documenting the presence and severity of the anemia, the strength of the bone marrow's response should be determined. Hemolytic anemias are usually the most regenerative (Table 24.4). Blood smear analysis frequently provides specific evidence of the cause of the hemolysis such as spherocytes,

autoagglutination, ghost cells, Heinz bodies, eccentrocytes or blood parasites. Icteric or hemolytic plasma or urinalysis with hemoglobinuria suggests hemolytic anemia, as does splenomegaly. If a regenerative anemia lacks indicators of hemolysis, then a blood loss anemia should be suspected. Macrocytic normochromic anemia is excluded from this veterinary algorithm because it has caused more misdiagnosis than correct diagnosis.

BLOOD LOSS ANEMIA

Blood loss can be external or internal (Table 24.5). Internal blood loss mimics hemolytic anemia in that erythrocytes are broken down within the body and plasma proteins are not lost from the body. External blood loss causes loss of plasma proteins as well as erythrocytes, so plasma protein concentration should be evaluated. Hypoproteinemia or low-normal plasma protein combined with regenerative anemia strongly suggests external blood loss. Plasma protein is replaced much quicker than erythrocytes so hypoproteinemia is more associated with recent and large volume blood loss. Evidence of bleeding comes from history, diagnostic imaging, fluid cytology, etc. Chronic blood loss causes iron deficiency anemia. Iron deficiency anemia due to bleeding occurs more quickly in young, growing animals that are rapidly producing erythrocytes and using iron stores.

Time after onset of blood loss greatly affects the pattern of laboratory results. Indicators of the erythroid mass in the body (Hct, Hgb concentration, RBC count) may even increase during the first few hours after blood loss. This is most prominent in horses with very contractile spleens but can be seen in dogs during controlled experimental situations.⁴ The spleen holds about 20% of the erythrocyte mass and splenic contraction can mask an anemia for the first 1–2 hours after blood loss.

The rapidity of fluid replacement and restoration of normal vascular volume after bleeding affects how rapidly remaining erythrocytes and plasma protein in the vascular system are diluted. It may take 1–2 days for the Hct to reflect the true severity of the anemia (Fig. 24.5). Withholding access to water can delay decreases in the Hct. Aggressive fluid therapy can reduce the Hct rapidly and even cause hemodilution.

Time affects the expected number of reticulocytes. Blood loss anemia is regenerative, but no increase in reticulocytes is expected for the first day or two during which it is termed a pre-regenerative anemia. Peak reticulocytosis is expected 4–8 days after onset (Fig. 24.5).⁶ Reticulocyte numbers decrease by 2 weeks after onset, but macrocytic hypochromic erythrocytes remain in circulation to give evidence of prior regeneration.

Blood loss anemia (Table 24.5) becomes less regenerative with time due to loss of nutrients like iron and protein. Chronic, severe blood loss causes iron deficiency anemia, which becomes progressively more non-regenerative (see Chapter 26). About half of iron deficiency anemia cases still have enough reticulocytosis to be called regenerative.

TABLE 24.3 Erythrocyte Shape Changes Associated With Selected Diseases or Conditions

Spherocyte	
Immune-mediated hemolytic anemias	Dog
Transfused blood	Dog
Blood parasite infections	Several species
Anaplasmosis	Cattle
Hereditary spherocytosis	Goat, cattle, dog
Zinc toxicity	Dog
Snake venom (coral snake, European viper)	Dog
Hypophosphatemia	Dog
Keratocyte/Shizocyte	
DIC	Dog, cat, cattle, horse
Congestive heart failure	Dog
Glomerulonephritis	Dog
Myelofibrosis	Dog
Hemangiosarcoma	Dog
Chronic doxorubicin toxicosis	Dog
Acanthocyte/Budding Fragmentation	
DIC	Dog
Hemangiosarcoma	Dog
Portosystemic shunts	Dog
Chronic liver disease	Dog, cat
Lymphosarcoma	Dog
Glomerulonephritis	Dog
Echinocyte	
Crenation artifact	Dog, cat, cattle, horse
Lymphosarcoma	Dog
Glomerulonephritis	Dog
Doxorubicin toxicity	Dog
Electrolyte depletion	Horse, dog
Heinz Bodies	
Onion, leek, garlic ingestion	Dog, cat, cow, horse, sheep
Vitamin K (mainly K ₃)	Dog
Acetaminophen treatment	Cat
Phenothiazine treatment	Horse
Red maple leaf ingestion	Horse
Methylene blue treatment	Dog, cat
Phenazopyridine treatment	Cat
Propylene glycol in food	Dog, cat
<i>Brassica</i> spp. ingestion	Cattle
Selenium deficiency	Cattle
Copper toxicity	Sheep
Zinc toxicity	Dog
Eccentrocytes/Pyknocyte	
Vitamin K treatment	Dog
Onion ingestion	Dog
Acetylphenylhydrazine	Dog
Hydrogen peroxide intravenously	Cattle
Glucose-6-phosphate dehydrogenase enzyme deficiency	Horse
Hypochromasia	
Iron deficiency	Dog, cat, cattle, horse, pig, llama, goats, birds
Inflammation	Birds
Lead toxicity	Birds
Macrocytosis	
Regenerative anemia	Many species
Poodle macrocytosis	Dog
Stomatocytosis	Dog
Feline leukemia virus infection	Cat
Myelodysplasia	Cat
Anticonvulsant (antifolate) drugs	Dog
Prolonged storage of blood	Many species
Hypernatremia	Cat, dog
Microcytosis	
Iron deficiency	Dog, cat, cow, horse, pig
Hepatic porto-systemic shunt	Dog
Hyponatremia	Dog
Normal breed characteristic	Dog (Akita, Shibas)

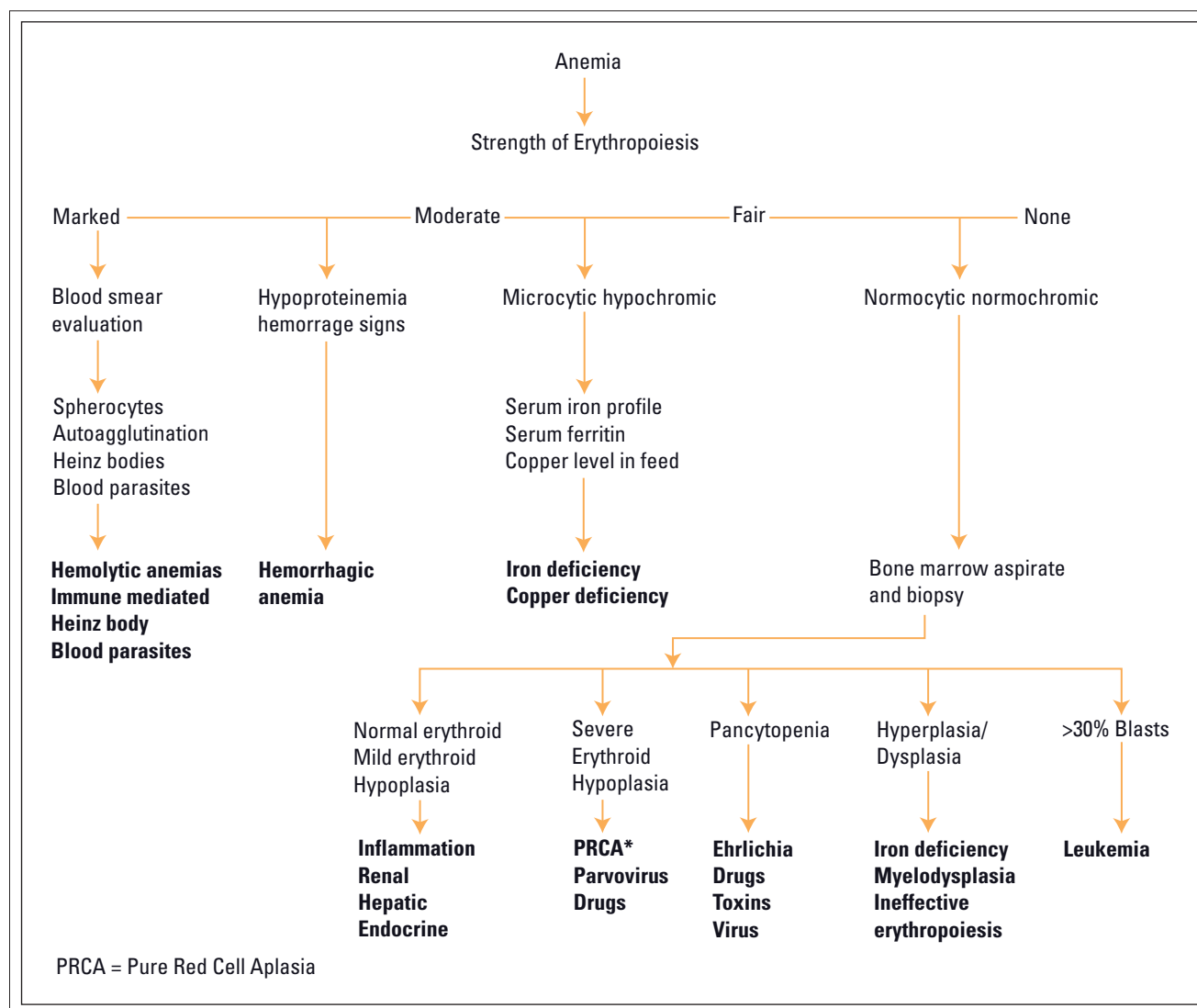


FIGURE 24.4 Algorithm for diagnosis of anemia. Anemia is documented and evaluated for severity. The strength of erythropoiesis is determined usually by reticulocyte evaluation. The marked to moderately regenerative anemias are usually hemolytic and hemorrhagic. Blood smear evaluation frequently identifies the type of hemolytic anemia. Diagnoses are given in bold. Hypoproteinemia and clinical signs indicate hemorrhagic anemia. Microcytic hypochromic anemia indicates iron deficiency that varies from non-regenerative to moderately regenerative. Non-regenerative and inadequately regenerative anemias with normocytic and normochromic RBCs may be diagnosed by bone marrow evaluation.

Confusion has occurred with interpretation of platelet counts during blood loss. A common misconception is that blood loss causes thrombocytopenia. This can mislead diagnosis of actual causes of thrombocytopenia. The platelet count is usually normal to increased after blood loss in dogs (Fig. 24.5B).²

NON-REGENERATIVE ANEMIA

The primary causes of secondary-types of mild to moderate non-regenerative anemia, such as inflammation, renal or hepatic disease, endocrine disease, neoplasia, etc., are diagnosed by tests more specific for those problems (e.g. leukogram, clinical chemistry, endocrinology,

cytology etc.). The cause of the anemia is usually clinically inferred from the type of disease present (e.g. if renal failure, then anemia of renal disease).

Bone Marrow Evaluation

Bone marrow evaluation is indicated in non-regenerative anemias that have had adequate time (e.g. 3–6 days) to respond, especially if there is a bicytopenia or pancytopenia present. Pancytopenia is a deficiency of cells in all three cell lineages and one should test for *Ehrlichia canis*, other bacteria, virus of that species, and check for exposure to various drugs and toxins.

Bone marrow evaluation is most complete when based on cytologic evaluation of aspirated smears, histologic evaluation of a cortex-to-cortex sections and a

TABLE 24.4 Selective Causes of Hemolytic Anemias

Immune-mediated	Idiopathic, viral (EIA), drugs, blood parasites, neonatal isoerythrolysis, transfusion reaction
Heinz body	Onions, vitamin K ₃ , phenothiazine, acetaminophen, phenacetin, acetanilide, phenylhydrazine-HCl, benzocaine, red maple, methylene blue, phenazopyridine, copper toxicity, selenium deficiency, <i>Brassica</i> sp. ingestion
Toxic hemolysis	Zinc, copper, water in calves, l-sorbose
Hypophosphatemia	Post-parturient hemoglobinuria, refeeding syndrome, diabetes mellitus
Blood parasites	<i>Anaplasma</i> , <i>Babesia</i> , <i>Haemobartonella</i> , <i>Eperythrozoon</i> , <i>Theileria</i> , <i>Trypanosoma</i> , <i>Sarcocystis</i> , <i>Cytauzoon</i>
Bacteria	<i>Leptospira</i> , <i>Clostridium novyi</i>
Hereditary RBC enzyme deficiencies	Pyruvate kinase, phosphofructokinase, glucose 6-phosphate dehydrogenase

TABLE 24.5 Selected Examples of Hemorrhagic Anemia

Trauma	Hit by car, penetrating wounds, surgical trauma
Bleeding lesions	Intestinal neoplasms, gastrointestinal tract ulceration, burns, infections
Parasites	Hookworms, coccidia, <i>Haemonchus</i>
	Fleas, lice
Hemostatic disorders	Thrombocytopenia, thrombopathy
Acquired coagulopathy (e.g. warfarin toxicity, hepatic disease)	
Hereditary coagulopathy, von Willebrand's disease	
Disseminated intravascular coagulation	

CBC on blood taken the same day as the bone marrow was obtained. Erythroid hypoplasia, pure red cell aplasia (PRCA), myelofibrosis, aplastic pancytopenia (fatty marrow) and leukemia are the clearest conclusions obtained in non-regenerative anemia.⁷ Severe decreases in only the erythroid line is called pure red cell aplasia and may be caused by immune-mediated damage or viruses such as parvovirus in dogs and FeLV in cats (see Chapter 38). Drugs and toxins cause a wide variety of effects on the bone marrow (see Chapters 16 and 17).

Ineffective erythropoiesis is a frequent occurrence. It is identified by finding an adequate to increased number of erythroid cells in the marrow but a non-regenerative anemia in the CBC. Causes of ineffective erythropoiesis include infections, drug reactions, myelodysplastic syndromes, and idiopathic causes. Erythroid hyperplasia and non-regenerative anemia occur in iron deficiency

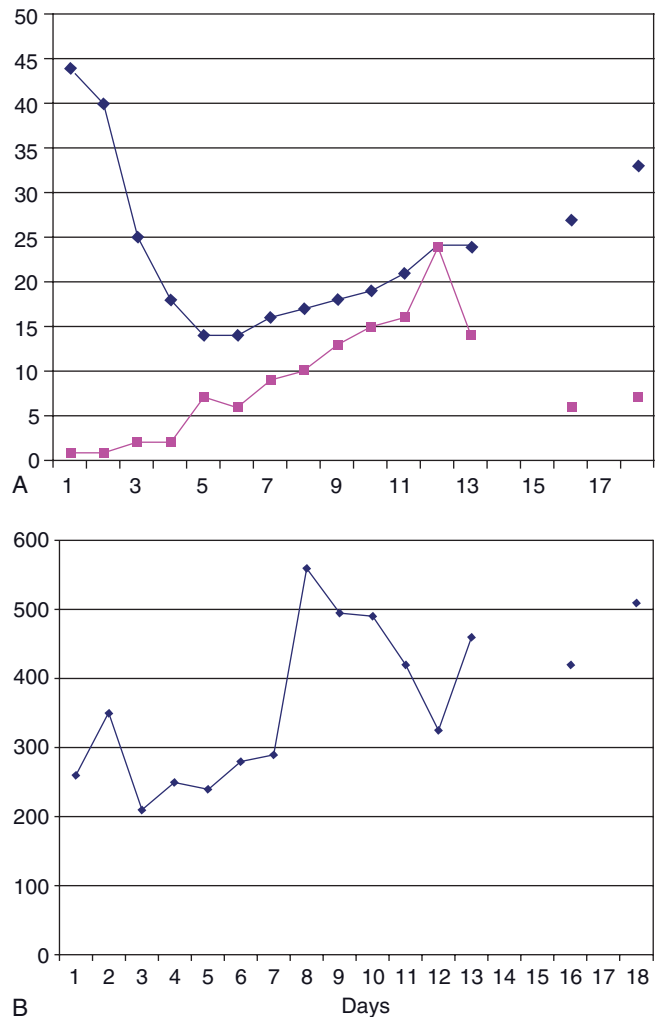


FIGURE 24.5 (A) Hematologic changes associated with canine blood loss anemia. A mature, healthy, research Beagle dog had 200–250 mL of blood removed each day for 3 consecutive days. An equal volume of saline and serum were infused intravenously to replace lost blood volume. These are days 2–4 in the figure.² Hematocrit (blue line with diamonds) and reticulocyte values (red line with squares) are given in percent on *y*-axis. Days are on the *x*-axis. Day 1 was the control value before bleeding. Note hematocrit does not reach its lowest values until days 5 and 6. The hematocrit was still low (33%) on day 18, which was 14 days after the last bleeding. Reticulocytes peaked at 24% on day 12, which was 8 days after the last bleeding and fell to 6–7% on days 16 and 18. (B) Thrombocytosis after canine blood loss. Platelet counts ($\times 10^9/L$) on the *y*-axis are from the same dog in Figure 24.5A. The dog had normal platelet counts before, during bleeding on days 2–4 and 3 days after the last bleeding. It had thrombocytosis on day 8 and higher than baseline platelet counts on days 9–18 which was 5–14 days after the last bleeding.

and will be accompanied by a lack of hemosiderin in macrophages.

Myelodysplastic syndromes are indicated by the presence of a hypercellular marrow, cytopenias, and abnormal cell morphology such as megaloblastic rubricytes, dwarf megakaryocytes, binucleated cells, nuclear fragments, and increased blast cells.

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Erythrocytosis and Polycythemia

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Relative Erythrocytosis

Absolute Erythrocytosis

Primary Erythrocytosis (Polycythemia Vera)

Secondary Erythrocytosis

Appropriate secondary erythrocytosis

Inappropriate secondary erythrocytosis

Endocrinopathy-associated erythrocytosis

Clinical Signs of Erythrocytosis

Diagnostic Approach

Treatment

Acronyms and Abbreviations

EPO, erythropoietin; P_{50} , the partial pressure of oxygen at which hemoglobin is half-saturated with oxygen; P_{aO_2} , arterial partial pressure of oxygen; PCV, packed cell volume; RBC, red blood cell; rPDA, reversed patent ductus arteriosus; S_{aO_2} , arterial hemoglobin oxygen saturation.

Red blood cell (RBC) production typically is in part regulated by the hormone erythropoietin (EPO; see Chapter 6). When oxygen sensors located in the renal cortex become hypoxic, hypoxia-inducible factors are generated and incite EPO gene transcription.^{23,43} The resultant EPO then stimulates erythroid precursors in the bone marrow to increase RBC numbers, thereby enhancing the oxygen carrying capacity of the blood.

Erythrocytosis is defined as an increase in peripheral RBC numbers, hemoglobin concentration, and calculated hematocrit or packed cell volume (PCV) above established reference intervals. Often, erythrocytosis is used interchangeably with the term polycythemia. However, in human medicine, polycythemia sometimes denotes not only erythrocytosis, but also concurrent increases in white blood cell and platelet counts.³⁸ Based on pathogenesis, erythrocytosis can be classified into relative or absolute categories. Absolute erythrocytosis can be further characterized as primary (polycythemia vera) or secondary (Fig. 25.1).

RELATIVE ERYTHROCYTOSIS

Relative erythrocytosis is the most common form of erythrocytosis in dogs and cats. In relative erythrocytosis, the increased PCV is not accompanied by an expanded RBC mass, but rather develops from diminished plasma volume and hemoconcentration due to fluid loss (i.e. severe dehydration associated with

vomiting, diarrhea, or polyuria without sufficient water intake). Another cause of relative erythrocytosis in dogs and horses is splenic contraction associated with excitement or anxiety. This catecholamine-mediated release of RBCs from their storage site in the spleen causes transient mild erythrocytosis unassociated with clinical signs.

ABSOLUTE ERYTHROCYTOSIS

Absolute erythrocytosis, defined as a true increase in RBC mass, can develop from primary or secondary causes.

Primary Erythrocytosis (Polycythemia Vera)

Primary erythrocytosis results from autonomous proliferation of erythroid precursors that require little to no EPO for differentiation. The prototypical primary erythrocytosis, also called polycythemia vera, is a chronic myeloproliferative disorder resulting from the clonal expansion of hematopoietic progenitor cells. In humans, polycythemia vera is associated with splenomegaly, leukocytosis, and thrombocytosis, and may progress to myelofibrosis or leukemia.^{25,38} However, many of those features are absent in dogs and cats with primary erythrocytosis. It remains unclear whether primary erythrocytosis in companion animals is the same disease as polycythemia vera in humans, or whether it represents a variant with isolated erythrocytosis. Regardless,

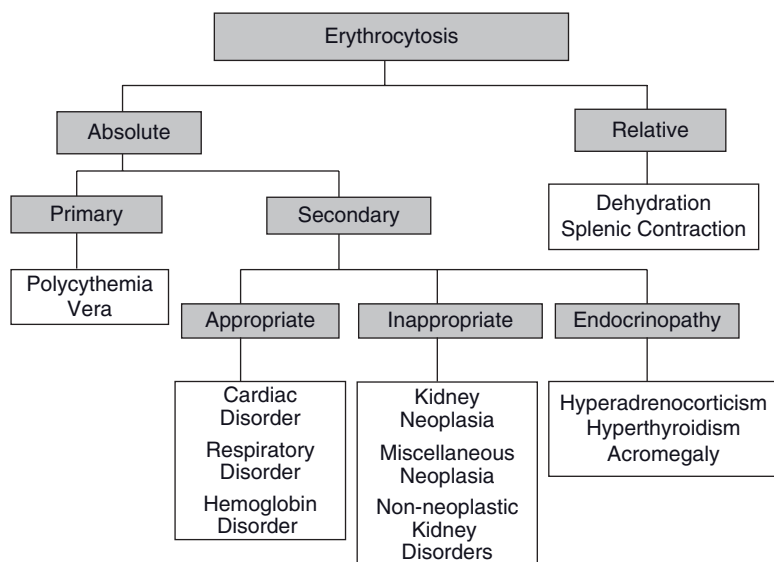


FIGURE 25.1 Flow chart for classification of erythrocytosis.

primary erythrocytosis is a disease of middle-aged (median, 6–7 years) dogs and cats.⁴⁹ Dogs with primary erythrocytosis are more frequently female, whereas affected cats are more commonly male.⁴⁹

Secondary Erythrocytosis

Secondary erythrocytosis develops from excessive production of EPO. If EPO is secreted in response to systemic hypoxia, then the resultant erythrocytosis represents an *appropriate* compensatory response. If, on the other hand, the increased EPO secretion is not associated with systemic tissue hypoxia, then the response is *inappropriate* (Fig. 25.1). Endocrinopathy-associated erythrocytosis is another type of secondary erythrocytosis resulting from hormonal (other than solely EPO-mediated) stimulation of erythropoiesis.

Appropriate Secondary Erythrocytosis

In physiologically appropriate erythrocytosis, the EPO-mediated increased RBC mass expands the oxygen carrying capacity of the blood in an attempt to improve inadequate tissue oxygenation. For example, congenital heart anomalies resulting in right-to-left shunting with blood bypassing the lungs (e.g. tetralogy of Fallot, reversed patent ductus arteriosus [rPDA]) cause delivery of poorly oxygenated blood to the systemic circulation with resultant erythrocytosis.^{4,5,16,17,24,28,31} Chronic severe pulmonary disease resulting in dyspnea from impaired oxygen transfer also may lead to erythrocytosis.^{10,37} Hereditary high-oxygen affinity hemoglobinopathies in humans cause decreased release of oxygen to tissue and consequent erythrocytosis.^{15,38} Although this type of hemoglobin disorder has not been described as a cause of pathologic erythrocytosis in companion animals, impaired oxygen transport from chronic methemoglobinemia in dogs and cats with methemoglobin reductase deficiency may cause mild erythrocytosis.¹⁹

Inappropriate Secondary Erythrocytosis

Excess production of EPO in the absence of systemic hypoxia, termed physiologically inappropriate erythrocytosis, is caused by: (1) EPO-secreting tumors of the kidneys or other organs, or (2) kidney disorders that result in regional hypoxia leading to increased EPO production. In humans, kidney neoplasms, as well as tumors of the liver, adrenal gland, uterus, and cerebellum, have been associated with inappropriate production of EPO and erythrocytosis.^{38,43} Tumor-related erythrocytosis has been described with renal carcinoma,^{8,36,47,48} renal lymphosarcoma,^{4,29} renal fibrosarcoma,¹⁸ nasal fibrosarcoma,⁶ cecal leiomyosarcoma,⁴¹ and schwannoma⁵² in the dog, and with renal carcinoma^{4,22} in the cat. Non-neoplastic kidney abnormalities in humans (e.g. renal cysts, hydronephrosis, glomerulonephritis)⁴³ and rarely in dogs (e.g. renal cryptococcosis)⁴⁸ have been reported as potential causes of erythrocytosis. For two dogs with glomerulonephropathy and erythrocytosis attributed to polycythemia vera, the kidney disorder was speculated to be a consequence, rather than a cause, of the erythrocytosis.^{32,39}

Endocrinopathy-Associated Erythrocytosis

Hormones other than EPO, such as cortisol, androgen, thyroxine, and growth hormone, also may stimulate erythropoiesis either directly, or indirectly through increased production of EPO or alternate pathophysiologic mechanisms.^{11,38} In dogs, increased cortisol or androgen concentrations associated with adrenal hyperactivity may produce mild erythrocytosis.¹¹ Similarly, cats with increased thyroxine concentrations from hyperthyroidism may have mildly increased RBC count.³⁵ Acromegalic cats with excess growth hormone may develop mild erythrocytosis,³⁵ but similar hematologic findings are usually absent in dogs with acromegaly.⁴⁰ The mild erythrocytosis in these endocrine

disorders is insufficient to result in clinical signs, and is usually discovered incidentally during routine diagnostic evaluation for the endocrinopathy.

CLINICAL SIGNS OF ERYTHROCYTOSIS

The clinical signs of both primary and secondary erythrocytosis include erythema (brick-red or ruddy color) of mucous membranes, neurologic disturbances (lethargy, ataxia, weakness, seizures, blindness, behavioral change), bleeding episodes (epistaxis, hematemesis, hematochezia, melena, hematuria), or polyuria and polydipsia. Most of these clinical manifestations are attributed to increased blood viscosity from the expanded RBC mass. In fact, the blood viscosity rises steeply as PCV increases.^{3,44} The hyperviscosity slows blood flow, distends capillaries and small vessels, may increase the likelihood of thrombosis and rupture of these vessels, and may impair tissue oxygen delivery.^{3,15,43} Vascular changes characteristic of hyperviscosity frequently can be visualized on ophthalmologic examination (i.e. dilated and tortuous retinal vessels, sometimes accompanied by retinal hemorrhage). The polyuria and polydipsia have been attributed to hyperviscosity and increased blood volume leading to impaired vasopressin release.⁴⁷

Laboratory test results may be skewed by extreme erythrocytosis. Hypoglycemia may be noted, and explained by enhanced glucose utilization by the increased number of RBCs in vivo or in vitro. Whole blood samples collected into sodium citrate anticoagulant tubes may result in prolonged prothrombin and activated partial thromboplastin times because of excess citrate concentration for the relatively smaller volume of plasma in patients with profound erythrocytosis.²³

DIAGNOSTIC APPROACH

Before an extensive diagnostic work-up is started, remember that many sighthound breeds, such as the Greyhound, normally have mild erythrocytosis when their PCV is compared to standard canine reference intervals.

In most animals, the first step in determining the reason for erythrocytosis is to exclude relative causes. Relative erythrocytosis usually results in a mild increase in PCV, rarely exceeding 60–65%.^{20,49} However, the PCV may be as high as 70–80% in some dogs with the hemorrhagic gastroenteritis syndrome due to rapid fluid loss into the gut. This observation emphasizes that the magnitude of increase in PCV does not necessarily differentiate between relative and absolute erythrocytosis. The dehydration and hemoconcentration of relative polycythemia usually are identified by clinical findings (e.g. dry mucous membranes, reduced skin turgor), laboratory variables (e.g. hyperproteinemia, pre-renal azotemia), and response to rehydration with intravenous fluid therapy. Excitable or anxious dogs with mild

erythrocytosis suspected to be caused by splenic contraction should have another blood sample collected under less stressful circumstances. Rarely, animals with repeatable mild increases in PCV and no obvious evidence of fluid loss may require direct measurement of RBC mass with radioisotope-labeled autologous RBCs to differentiate relative from absolute causes. However, these studies seldom are performed in clinical practice.

Once relative erythrocytosis is excluded, the next step is to differentiate primary and secondary causes of absolute erythrocytosis. Traditionally, serum EPO determinations have been recommended to distinguish these two types. In secondary erythrocytosis, increased EPO activity would be expected, whereas such activity should be low in primary erythrocytosis. However, recent studies have demonstrated considerable overlap in EPO activity among normal animals, animals with primary erythrocytosis, and animals with secondary erythrocytosis.^{4,21} Fluctuations in EPO secretion, production of another hematopoietic growth factor not detected by the EPO assays, or renal hypoxia secondary to hyperviscosity may explain these findings.^{4,25} Repeat EPO determinations, even following phlebotomy, have been suggested in problematic cases.^{2,14,15,23,45} Even though most dogs and cats with primary erythrocytosis have low to low-normal EPO activity, and many animals with secondary erythrocytosis have higher EPO values, results outside of these expected ranges have been encountered.^{2,4,14,21} For that reason, EPO determinations should complement rather than supplant a thorough diagnostic evaluation of animals with erythrocytosis. Furthermore, current availability of validated EPO assays for dogs and cats is limited. Additional efforts to match diagnostic features of polycythemia vera in humans with primary erythrocytosis in companion animals have been unrewarding. Routine examination of bone marrow in affected dogs and cats cannot diagnose or differentiate primary and secondary erythrocytosis because both conditions are characterized by erythroid hyperplasia. Therefore, a diagnosis of primary erythrocytosis typically is made by excluding common secondary causes.

Arterial blood gas analysis including determination of partial pressure of oxygen (P_{aO_2}) and hemoglobin oxygen saturation (S_{aO_2}) is useful in assessing tissue oxygenation. A P_{aO_2} less than 80 mmHg indicates hypoxemia and S_{aO_2} less than 95% reflects tissue hypoxia, both values warranting consideration of appropriate secondary erythrocytosis. If initial collection of arterial blood is thwarted by hyperviscosity, sampling may be repeated following cautious phlebotomy.²⁰ Alternatively, capillary S_{aO_2} may be measured non-invasively with a pulse oximeter. Examination of cardiac and pulmonary systems by thoracic auscultation, thoracic radiographs, electrocardiography, and echocardiography may help determine the cause of appropriate erythrocytosis. Studies to confirm a right-to-left cardiac shunt include selective angiography and contrast echoaortography.^{12,17} In dogs with rPDA, cyanosis of the vulvar or preputial (but not the oral) mucous membranes may be detected

at rest or following exercise; similarly, lower P_{aO_2} values may be identified at the tail or hindlimbs compared to head or forelimbs.¹² This differential hypoxia in rPDA develops because deoxygenated blood shunts from the pulmonary artery into the aorta distal to the left subclavian artery and brachycephalic trunk, allowing more oxygenated blood to travel to the head and forelimbs. Abnormal hemoglobin variants with increased oxygen affinity in humans can be recognized by demonstrating normal P_{aO_2} and S_{aO_2} values, but reduced partial pressure of oxygen when hemoglobin is half-saturated with oxygen (P_{50}).^{20,43} These hemoglobinopathies have yet to be confirmed as a cause of pathologic polycythemia in companion animals. However, some investigators have proposed that the higher PCV (and lower hemoglobin P_{50}) in Greyhounds may indicate that the hemoglobin of this breed has a greater affinity for oxygen.⁴⁵

Once systemic hypoxia is excluded as the cause of erythrocytosis, conditions causing inappropriate EPO production should be considered. To locate the source of EPO in these disorders, results of physical and neurologic examinations, abdominal ultrasonography, intravenous urography, and computed tomography or magnetic resonance imaging should be helpful.

TREATMENT

With relative erythrocytosis due to dehydration, fluid therapy should be administered and the underlying disorder should be addressed. The PCV will usually normalize with successful management. For relative erythrocytosis resulting from splenic contraction, no treatment is necessary.

In primary erythrocytosis, the goal of therapy is to reduce and maintain the PCV within reference intervals, thereby alleviating clinical signs and preventing any potential thrombotic and hemorrhagic complications. The optimal treatment, however, is unknown. In dogs and cats, the disease has been managed with phlebotomy alone^{13,26,27} or in combination with other modalities, leeching³⁰, radioactive phosphorus^{26,42,51}, or chemotherapeutic agents such as doxorubicin³² and busulfan²⁶. Phlebotomy and leeching must be repeated on a regular basis to maintain the PCV within reference intervals and can lead to iron deficiency and hypoproteinemia. Radioactive phosphorus induces prolonged remission and reduces morbidity, but is not readily available and may increase the risk of leukemia in treated humans.^{23,38} Some of the chemotherapeutic drugs used to treat humans with polycythemia vera also may be leukemogenic.^{23,38} However, it remains unknown whether leukemic transformation in these instances represents complications of treatment or natural progression of this chronic myeloproliferative disease.^{23,38} The infrequent development of leukemia in dogs and cats with primary erythrocytosis may be further proof that it is indeed distinct from polycythemia vera in humans, or may merely reflect the prolonged time (10–15 years) needed for leukemic transformation in humans.²³

Hydroxyurea, an anti-neoplastic drug, is most commonly used to manage primary polycythemia in dogs and cats. Side effects of hydroxyurea include anorexia, vomiting, bone marrow hypoplasia, mild alopecia, and sloughing of the nails.³⁴ Cats also may develop methemoglobinemia and hemolytic anemia with Heinz bodies, especially with high doses.⁵⁰ The effects of hydroxyurea are enhanced by reducing the PCV to approximately 50–60% with phlebotomy (10–20 mL/kg) and simultaneous fluid replacement.^{33,34} Then, hydroxyurea is given at a loading dose of 30 mg/kg PO daily for 7–10 days, followed by 15 mg/kg PO daily in single or divided doses.^{33,34,46} Alternate dosing schedules for cats have been reported.^{7,9,50} Complete blood and platelet counts should be monitored at 7–14 day intervals until the PCV has normalized, then every 1–3 months. If leukopenia, thrombocytopenia, or anemia develops, hydroxyurea should be discontinued until the blood count returns to normal, and then resumed at a lower maintenance dosage or dose frequency.^{33,34}

In inappropriate secondary erythrocytosis, treatment should be directed toward the underlying cause, when feasible. Erythropoietin-secreting tumors should be surgically removed if practical, or managed with chemotherapy and/or radiation therapy. Pre-operative phlebotomy to normalize the PCV may help reduce morbidity and mortality.

In appropriate erythrocytosis secondary to systemic hypoxia, as in some cardiac diseases, treatment of the underlying problem is recommended, if possible. Unfortunately, the two most common types of congenital heart defects associated with polycythemia in pets are not easily amenable to surgical correction: rPDA cannot be corrected surgically because of the attendant pulmonary hypertension, and mortality is high for attempted surgical correction of tetralogy of Fallot.¹⁶ If correction is not possible, then long-term maintenance of the desired PCV can be accomplished with cautious phlebotomy (5–10 mL/kg) and hydroxyurea therapy as outlined for primary erythrocytosis: alternate protocols using hydroxyurea also have been successful.²⁸ However, because the erythrocytosis in these cases represents a compensatory response to tissue hypoxia, the PCV should be maintained between 55% and 60% rather than attempting normalization.² This degree of erythrocytosis seemingly is sufficient to minimize clinical signs associated with the systemic hypoxia as well as the polycythemia-associated hyperviscosity. Other investigators have recommended maintaining the PCV at even higher values (62–68%), or simply relieving clinical signs without concern about attaining a specific PCV.^{1,5,38} Regardless, phlebotomy and drug administration in these patients should be performed with careful monitoring.

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Iron and Copper Deficiencies and Disorders of Iron Metabolism

DOUGLAS J. WEISS

Tests for Evaluation of Iron Disorders

- Erythrocyte Indices
- Serum Iron
- Serum Total Iron Binding Capacity
- Serum Ferritin
- Bone Marrow Iron
- Erythrocyte Zinc Protoporphyrin

Iron Deficiency

- Causes
- Clinical Features
- Laboratory Features

Functional Iron Deficiency

- Anemia of Inflammatory and Neoplastic Diseases (see Chapter 37)
- Portosystemic Shunts
- Other Microcytic or Hypochromic Anemias
- Refractory Anemia with Ringed Sideroblasts (see Chapter 64)
- Sideroblastic Anemias
- Copper Deficiency
- Iron Overload
 - Acute Iron Toxicity
 - Hemosiderosis and Hemochromatosis
 - Primary hemochromatosis
 - Secondary hemochromatosis

Acronyms and Abbreviations

MCHC, mean cell hemoglobin concentration; MCV, mean cell volume; RBC, red blood cell; RDW, red blood cell distribution width; TIBC, total iron binding capacity.

Iron is an essential component of all living organisms, playing a role in many metabolic processes particularly those involving electron transfer. Copper is a component of several metalloproteins including cytochrome c oxidase, superoxide dismutase, urate oxidase, dopamine β -hydroxylase, ascorbic acid oxidase, and tyrosine oxidase. Iron absorption, metabolism, and utilization in erythropoiesis are discussed in Chapter 20. In this chapter disorders of iron and copper metabolism will be discussed.

TESTS FOR EVALUATION OF IRON DISORDERS

Erythrocyte Indices

Iron is required for hemoglobin synthesis (see Chapter 20). Both true and functional iron deficiency result in

deficient hemoglobin synthesis. Because hemoglobin concentration signals cessation of cell division, iron deficient cells undergo one or two more cell divisions resulting in formation of microcytes.²⁸ Red blood cells may also be hypochromic. Both MCV and MCHC tend to be decreased in iron deficiency but in some cases MCHC may be normal when the MCV is mildly decreased.

Many flow cytometer-based hematology analyzers can determine and plot the volume and hemoglobin content of individual RBCs (see Chapter 135). This technology can detect low numbers of microcytic or hypochromic RBCs when the MCV and MCHC are within reference intervals. These cells can be detected by inspection of cytograms. The percentage of microcytes or hypochromic or hyperchromic RBCs can be quantified for both mature RBCs and reticulocytes. Because reticulocytes have been recently produced, they most accurately reflect the current iron status.²⁷

Serum Iron

Serum iron assesses the transport compartment (see Chapter 147). Serum iron is typically low in iron deficiency and in inflammatory diseases.^{10,31} Serum iron may also be low during accelerated erythropoiesis particularly when exogenous erythropoietin is administered without iron supplementation.² Serum iron concentration is also decreased by glucocorticoid administration to ruminants.²⁹ Serum iron is typically increased in hemolytic anemias, dyserythropoiesis, hypoplastic anemia, aplastic anemia, iron overload, and in dogs and horses given glucocorticoids.^{11,25,34}

Serum Total Iron Binding Capacity

The total iron binding capacity (TIBC) is a measure of the total serum transferrin concentration (see Chapter 146). TIBC is increased in iron deficiency in humans, horses, cattle, and pigs, but does not appear to be increased in clinical iron deficiency in dogs.¹⁰ TIBC is decreased or in the low-normal range in inflammatory diseases.³

Serum Ferritin

Despite being an acute phase protein, serum ferritin correlates with tissue iron stores in domestic animals.²⁶ Increased serum ferritin occurs in animals with increased iron stores associated with chronic hemolytic anemias, and repeated blood transfusions. Other causes of very high serum ferritin concentrations include benign hemophagocytic disorders and malignant histiocytosis. Serum ferritin is also transiently increased in horses by intense exercise. Serum ferritin is decreased in animals with iron deficiency.

Bone Marrow Iron

Bone marrow aspirates or core biopsies can be stained with Prussian blue stains to detect hemosiderin. Hemosiderin is a tissue storage form of iron and stains blue when the Prussian blue stain is applied. Bone marrow aspirates used for Prussian blue staining should contain several unit particles so adequate material is present for assessment of marrow iron. Hemosiderin is stored within macrophages. Animals with iron deficiency lack stainable iron in bone marrow; however, lack of stainable iron is not necessarily predictive of iron deficiency.⁵ Healthy non-iron deficient cats and some cattle lack stainable iron in bone marrow.⁸

Erythrocyte Zinc Protoporphyrin

Heme is formed by the addition of reduced iron to protoporphyrin IX. The reaction is catalyzed by ferrochelatase. When iron availability is reduced, zinc becomes a substrate for ferrochelatase leading to the formation of zinc protoporphyrin. Zinc protoporphyrin is stable and remains in RBCs throughout their lifespan. Zinc protoporphyrin can be measured in washed RBCs

using a hematofluorometer. Due to the long lifespan of erythrocytes, iron deficiency must exist for an extended period to permit zinc protoporphyrin concentrations to exceed the reference interval.¹⁸ RBC zinc protoporphyrin concentrations are high in iron deficiency and in chronic inflammatory diseases. In inflammatory diseases iron is sequestered in the mononuclear phagocyte system and is, therefore, unavailable for erythropoiesis.⁴ RBC zinc protoporphyrin concentration also increases in lead poisoning.¹⁸

IRON DEFICIENCY

Causes

True iron deficiency is caused by insufficient gastrointestinal iron absorption or by hemorrhage. Iron deficiency due to decreased intake is rare except in neonatal animals that are nursing. Milk contains low concentrations of iron; therefore, nursing animals can easily become iron deficient as they grow.¹⁵ Piglets reared without access to soil are highly susceptible to iron deficiency. Concurrent infestations with bloodsucking parasites can result in severe iron deficiency. Iron deficiency in piglets can be prevented by allowing them to eat soil or by intramuscular iron dextran injections.

Iron deficiency is a relatively common finding in ruminants and dogs in temperate regions where bloodsucking parasite infestations are severe.¹⁰ Other causes of chronic blood loss include gastrointestinal ulcers, gastrointestinal neoplasia, hemorrhagic colitis, urinary bladder neoplasia, thrombocytopenia, and inherited hemostatic disorders. Iron deficiency is rarely seen in adult horses or cats.²⁴

Clinical Features

Iron deficiency has been classified into three stages: iron deficiency, iron-deficient erythropoiesis, and iron deficiency anemia.¹³ In iron deficiency, bone marrow iron and serum ferritin are low but serum iron, transferrin saturation, zinc protoporphyrin, and complete blood count values are normal. In iron-deficient erythropoiesis, bone marrow iron and serum ferritin and serum iron transferrin saturation are low, zinc protoporphyrin is high, but complete blood count values remain within the reference intervals. In iron deficiency anemia, bone marrow iron and serum ferritin and serum iron transferrin saturation are low, zinc protoporphyrin is high, and complete blood count values, including hemoglobin, MCV, and MCHC, are altered.

Iron deficiency is usually not identified until a microcytic anemia is present. Clinical signs associated with iron deficiency vary somewhat depending on the cause of the iron deficiency and the presence of other concomitant disorders. Clinical signs that may be present include pale mucous membranes, lethargy, weakness, loss of weight, retarded growth, hematuria, dermatitis, hematochezia, and melena. These signs appear to arise not only from the associated anemia but from deficiency of other iron-containing molecules including cyto-

chrome c, cytochrome oxidase, succinic dehydrogenase, aconitase, xanthine oxidase, and myoglobin.¹⁵ Iron-deficient animals have increased susceptibility to infection; however, the mechanism involved is poorly understood.¹⁵

Laboratory Features

The anemia in iron deficiency is complex, usually resulting primarily from a combination of hemorrhage and impaired erythropoiesis. However, RBCs produced in an iron-deficient environment also have a reduced lifespan.¹⁴ In acute iron deficiency anemia, the anemia is normocytic and normochromic. The MCV drops below the reference interval only after weeks to months of iron deficiency. In iron deficient animals, a decrease in MCV usually precedes a decrease in MCHC. A low MCHC is frequently present in chronic iron deficiency in ruminants and dogs but is rarely present in iron deficient horses or adult cats. The RBC distribution width (RDW) is frequently increased because of the coexistence of microcytes and normocytes in the blood. Acute iron deficiency is under diagnosed because in many cases the anemia is normocytic and normochromic. However, careful evaluation of RBC cytograms and reticulocyte indices, as well as determination of the percentage of microcytes, provides much earlier indicators of iron deficiency anemia (see Chapter 135). This technology can detect low numbers of microcytic or hypochromic RBCs when the MCV and MCHC are within the reference range. Because reticulocytes are recently produced, reticulocyte indices ($MCV_{retic} \times CH_{retic}$) most accurately reflect the current iron status.²⁷

Alteration in RBC morphology in blood smears is most prominent in dogs and ruminants. In these species hypochromic RBCs have a narrow rim of lightly stained hemoglobin and a larger than normal area of central pallor (Fig. 26.1; see Chapter 23). Because of the increased diameter-to-volume of these cells they tend to flatten on the slide and therefore may not appear small when viewed on the stained blood film. Hypochromasia is less apparent in blood films from cats and horses. Further, electronic cell counters may not count microcytic RBCs due to their small size. This results in a spuriously increased MCV. Poikilocytosis, including keratocytes and schistocytes, is frequently present in iron deficiency. The mechanism responsible for these shape alterations is uncertain.

When iron deficiency anemia is the result of hemorrhage, reticulocytosis is frequently present in the early stages. As iron depletion becomes more severe erythropoiesis is compromised and the reticulocytosis is abrogated. Moderate to marked thrombocytosis is a frequent finding in iron deficiency. The mechanism is not clearly defined.

FUNCTIONAL IRON DEFICIENCY

In functional iron deficiency, total body iron stores are normal or increased but iron is relatively unavailable for heme synthesis. Functional iron deficiency has been

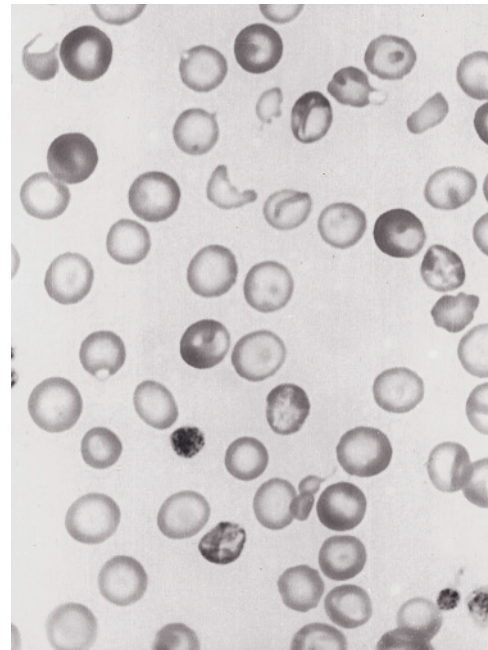


FIGURE 26.1 Hypochromasia and poikilocytosis in a blood smear from a dog with chronic iron deficiency anemia. Wright-Giemsa stain.

associated with the anemia of inflammatory and neoplastic diseases (see Chapter 37) and portosystemic shunts.

Portosystemic Shunts

Portosystemic shunts are vascular connections between the portal and systemic circulation that divert blood around the liver. These vessels may occur congenitally or arise secondary to portal hypertension associated with chronic hepatic disease. Most affected dogs have mild microcytosis and hypochromasia but are not anemic.¹⁹ Poikilocytosis, including codocytes, keratocytes, and elliptocytes, are frequent findings. Approximately half of the affected dogs have hypoferrremia but serum ferritin is high.¹⁹

OTHER MICROCYTIC OR HYPOCHROMIC ANEMIAS

Microcytic or hypochromic anemias have been associated with sideroblastic anemia and refractory anemia with ringed sideroblasts. Refractory anemia with ringed sideroblasts is a type of myelodysplastic syndrome (see Chapter 66).⁶

Sideroblastic Anemias

Sideroblastic anemias result from disorders of heme synthesis which result in accumulation of excess iron in mitochondria of erythroid cells. The hallmark of sideroblastic anemias is the present of large numbers of iron aggregates within RBCs (i.e. sideriocytes) or erythroid precursors (sideroblasts) or the presence of iron

distributed in a ring around the nucleus of erythroid precursor cells (i.e. ringed sideroblasts). Naturally occurring pyridoxine deficiency in humans and experimental pyridoxine deficiency in pigs results in sideroblastic anemia.⁷ Pyridoxine is required for the first step in heme synthesis. Several causes of sideroblastic anemia have been reported in dogs. Drugs and chemicals associated with sideroblastic anemia include chloramphenicol, hydroxyzine, zinc, and lead.^{9,12} Sideroblastic anemia associated with clinical disease has primarily been seen in dogs with inflammatory diseases.³⁰ The hemogram in these dogs is characterized by moderate to severe non-regenerative anemia that is frequently hypochromic and the presence of dysplastic features in bone marrow that is most prominent in the erythroid lineage. Siderocytes and sideroblasts and variable numbers of ringed sideroblasts are a prominent feature of this condition.³²

COPPER DEFICIENCY

Copper deficiency can develop in ruminants eating forage from copper deficient soils. Secondary copper deficiency can occur when copper intake is adequate but use is impaired by high dietary molybdenum or sulfate. Affected animals generally have a microcytic hypochromic anemia; however, normocytic anemias have been reported in experimental copper deficiency in dogs.¹ Microcytosis and hypochromasia is the result of functional iron deficiency. Functional iron deficiency results from a decrease in production of the copper-containing proteins ceruloplasmin, hephaestin, and cytochrome oxidase that are required for iron transport.¹⁶ Hephaestin is involved in release of iron from intestinal enterocytes. Ceruloplasmin facilitates mobilization of iron from the liver and tissues other than enterocytes.³³ Pigs with acute experimental copper deficiency have low serum iron but pigs with chronic experimental copper deficiency develop increased serum iron concentration and have many sideroblasts present.¹⁶ This may be due to a deficiency of the copper-containing enzyme cytochrome oxidase in erythroid precursor cells. Cytochrome oxidase is involved in reducing ferric to ferrous iron in mitochondria for use in heme synthesis.

IRON OVERLOAD

Iron overload can result from acute iron toxicity or chronic accumulation that results in hemosiderosis or hemochromatosis. Iron accumulates in the liver and other tissues when transferrin saturation is high. Although most iron is stored as ferritin, some is likely available to catalyze the formation of reactive oxygen species.²²

Acute Iron Toxicity

Acute iron toxicity usually results from administration of large doses of iron to animals. Parenteral administra-

tion of large amounts of iron to ruminants results in respiratory distress, icterus, and central nervous system signs.²³ Oral intake of a large amount of iron results in respiratory distress, icterus, central nervous system signs, vomiting, bloody diarrhea, and hypovolemic shock. Hepatotoxicity has been documented in neonatal foals after ingestion of a nutritional supplement containing ferrous fumarate iron at a concentration lower than that required to induce acute iron toxicity in adult horses.²⁰

Hemosiderosis and Hemochromatosis

Hemochromatosis refers to accumulation of iron in tissue with associated organ injury and dysfunction, while hemosiderosis refers to accumulation of iron within tissues without evidence of organ injury or dysfunction. Hemosiderosis occurs locally within tissues at sites where hemorrhage has occurred. Hemochromatosis has been classified as primary (inherited), secondary, and idiopathic.¹⁷

Primary Hemochromatosis

Most types of hereditary hemochromatosis in humans result from a mutation in the hepcidin gene. Hemochromatosis has also been described in related Salers cattle.²¹ Affected cattle had a history of stunted growth, poor hair coat, and weight loss. These cattle had iron accumulation in multiple tissues and histopathologic lesions consisting of periportal fibrosis, periosteal dysplasia, and osteopenia.

Secondary Hemochromatosis

Secondary hemochromatosis is most frequently associated with hemolytic anemias, repeated blood transfusions, parenteral iron administration, or dietary iron overload. Dietary iron overload does not appear to be a problem in animals except in a few wild animal species housed in captivity.¹⁷ Hemolytic anemias are associated with increased iron absorption. When the hemolysis is chronic, increased intestinal absorption results in iron accumulation within hepatocytes as well as within macrophages. Liver fibrosis eventually develops.

Iron overload associated with blood transfusions is rarely a problem in animals. In general, large numbers of transfusions can be given before hepatocellular degeneration or fibrosis is detected.

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The Porphyrins and Porphyrinurias

J. JERRY KANEKO

Porphyrin Synthesis: the Porphyrins and the Porphyrinurias

Diagnosis of the Porphyrins and the Porphyrinurias

Clinical Observations

Laboratory Detection

Classification

Erythropoietic Porphyrins

Bovine Congenital Erythropoietic Porphyria

Clinical observations

Laboratory detection

Detection of the carrier state

Bovine Erythropoietic Protoporphyrin

Porphyria of Swine

Porphyria of Cats

Normal Porphyrias

Hepatic Porphyrias

δ -Aminolevulinic Acid Dehydratase Porphyria

Acute Intermittent Porphyria

Porphyria Cutanea Tarda

Hepatoerythropoietic Porphyria

Harderoporphyria

Hereditary Coproporphyrin

Variegate Porphyria

Acquired Toxic Porphyrias: the Porphyrinurias

Chemical Porphyrias

Lead Poisoning

Acronyms and Abbreviations

ADP, ALA-D porphyria; AIP, acute intermittent porphyria; ALA-syn, δ -aminolevulinic synthetase; ALA-D, ALA-dehydrase; CEP, congenital erythropoietic porphyria; DHC, dihydrocollidine; EPP, erythropoietic protoporphyria; FER-Ch, protoporphyrin IX by ferrochelatase; GGT, γ -glutamyltransferase; HCB, hexachlorobenzene; HCP, hereditary coproporphyrin; HEP, hepatoerythropoietic porphyria; NRBC, nucleated erythrocytes; 5'NT, pyrimidine-5'-nucleotidase; PCT, porphyria cutanea tarda; RPI, reticulocyte production index; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; UV, ultraviolet.

The diseases associated with the accumulation of porphyrin compounds in cells, tissues, and body fluids are classified as the porphyrias or the porphyrinurias. The porphyrias are those inherited enzyme defects of the metabolic pathway leading to the synthesis of porphyrins and their metal complexes, the hemoglobins, other heme proteins and the heme enzymes.¹ The porphyrinurias are those acquired defects due to chemical and metal toxicities of the same pathway of porphyrin synthesis. The heme proteins are found widespread in nature. The photosynthetic pigment of plants, chlorophyll, is a magnesium-porphyrin. The heme proteins of animals include the hemoglobins, myoglobins and heme enzymes such as catalase, peroxidase and cytochromes. As such, the heme compounds are vital to the capture and delivery of oxygen to tissues and to the subsequent generation of chemical energy to sustain life. This chapter will focus on heredi-

tary and acquired events in which toxic porphyrin compounds accumulate amid failures to synthesize the precise porphyrin structures required for their incorporation into functional heme proteins.

PORPHYRIN SYNTHESIS: THE PORPHYRIAS AND THE PORPHYRINURIAS

The biochemical pathway for the synthesis of porphyrin and the heme of hemoglobin is summarized in Chapter 20 and are outlined in Fig. 27.1 and Table 27.1.¹⁰ The synthesis of porphyrins and heme can occur only in respiring cells with a full complement of mitochondrial and cytosolic enzymes. The Krebs cycle is an aerobic cycle, so oxygen would be required in order to supply the succinyl-CoA. The predominant tissues that synthesize heme are the bone marrow for production of

hemoglobin and the liver for the heme-containing enzymes. Thus, these are the two tissues that are central to the pathogenesis of the porphyrias and the porphyrinurias.

DIAGNOSIS OF THE PORPHYRIAS AND THE PORPHYRINURIAS

Clinical Observations

Clinically, the detection of the porphyrins is based upon their reddish-brown color, their characteristic red fluo-

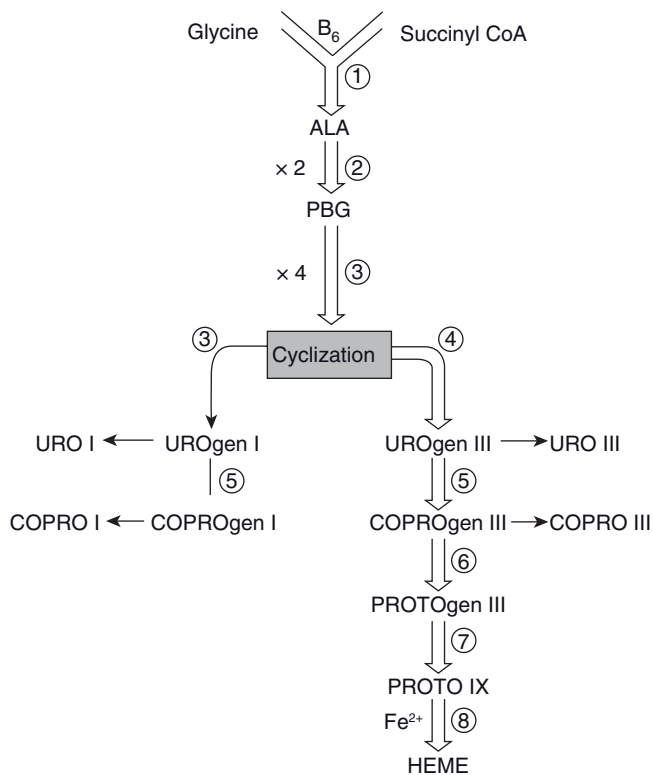


FIGURE 27.1 Alternate pathways for porphyrin synthesis. Normally, enzymes 3 and 4 function together in a coordinated manner to form heme. In the absence of enzyme 4, the alternate and terminal pathway to form the I isomers is taken. The circled numbers correspond to the enzymes listed in Table 27.1.

rescence when exposed to ultraviolet (UV) light and the lesions of photosensitivity due to the photosensitizing nature of the porphyrin compounds. Because they are present in all body cells, tissues and fluids, the porphyrins stain all tissues. This is particularly noticeable in the reddish-orange staining of the teeth and dentine, hence the name "pink tooth" is colloquially used for congenital erythropoietic porphyria (CEP). In well vascularized tissues (e.g. mucous membranes) a muddy color is readily detected. Porphyrins in the body fluids are readily excreted in the urine and in saliva, sweat, tears, and mucous secretions. The reddish-orange discoloration of the urine is readily visible and is often the presenting complaint. When UV light of the Soret band (405nm) or a Wood's lamp is shone on the teeth or urine, an intense reddish fluorescence is seen. This is virtually pathognomonic of porphyria but this red fluorescence should not be confused with the yellow-green fluorescence of tetracycline drugs. The third major clinical observation is photodermatitis, which is evident on light colored areas of the skin. This may be accompanied by photophobia. When exposed to UV light, the porphyrins in the skin absorb UV light energy and are raised to an unstable higher level energy state. The excitation energy can be emitted as fluorescence or transferred to molecular oxygen to form singlet oxygen. Singlet oxygen is a powerful oxidant for many forms of biologically important compounds including the peroxidation of membrane lipids, membrane and cellular proteins, cell enzymes and cell organelles. Peroxidation is the primary event in the photosensitivity and photodermatitis seen in the porphyrias.¹⁶

Laboratory Detection

The principal method now employed for the detection of porphyrins in biological materials in the clinical laboratory is also based upon the characteristic red fluorescence observed when acidic solutions of the porphyrins are exposed to UV light.

Fluorescence does not differentiate the uroporphyrins from the coproporphyrins so these are separated on the basis of their solubilities in organic solvents prior to exposure. Various screening and chemical procedures are described in detail for urine, fecal and blood

TABLE 27.1 Nomenclature for Enzymes of Porphyrin and Heme Synthesis and their Synonyms

No. in Figure 27.1	Abbreviation	Nomenclature
1	ALA-Syn	δ -Aminolevulinate synthase (synthetase)
2	ALA-D	δ -Aminolevulinate dehydrase (dehydratase), porphobilinogen synthase
3	UROgenI-Syn	Uroporphyrinogen I synthase (synthetase), porphobilinogen deaminase
4	UROgenIII-Cosyn	Uroporphyrinogen III cosynthase (cosynthetase)
5	UROgen-D	Uroporphyrinogen decarboxylase
6	COPROgenIII-Ox	Coproporphyrinogen III oxidase
7	PROTOgen-Ox	Protoporphyrinogen oxidase
8	FER-Ch	Ferrochelatase, heme synthase (synthetase)

TABLE 27.2 Classification of the Porphyrins

Porphyria Type	Inheritance ^a	Enzyme Deficiency ^b
Erythropoietic porphyrias		
Congenital erythropoietic porphyria	AR	UROgenIII-Cosyn
Erythropoietic protoporphyria	AD	FER-Ch
Hepatic porphyrias		
ALA-D deficiency porphyria	AR	ALA-D
Acute intermittent porphyria	AD	UROgenI-Syn (PBG-D)
Porphyria cutanea tarda	AD	UROgen-D
Hepatoerythropoietic porphyria	AR	UROgen-D
Harderoporphyria	AR	COPROgenIII-Ox
Hereditary coproporphyria	AD	COPROgenIII-Ox
Variagate porphyria	AD	PROTOgen-Ox

^aA, autosomal; R, recessive; D, dominant.

^bSee Table 27.1 for enzyme abbreviations.

porphyrins as well as for urinary porphobilinogen.¹⁰ High pressure liquid chromatography is now regarded as the best chemical method for detection of porphyrins in feces and in urine.^{27,28}

CLASSIFICATION

The porphyrias are those porphyrin diseases that are inherited. There have been many classification systems for the porphyrias. Most are based on the defect in the tissue of origin, the erythropoietic system or the liver.^{22,23} A useful system of classification is given in Table 27.2. The porphyrinurias are acquired porphyrin diseases and are generally due to toxins. Coproporphyrin is the main excretory product and the porphyrinurias are seen in a wide variety of conditions including infections, hemolytic anemias, liver disease, and lead poisoning. There are other systems of classification of the porphyrias and while there is general agreement on the erythropoietic forms, there is still some confusion with the classification of the hepatic forms.

ERYTHROPOIETIC PORPHYRIAS

Bovine Congenital Erythropoietic Porphyria

Clinical Observations

The most characteristic finding in bovine CEP is a reddish brown discoloration of the teeth and bones. The disease is seen primarily in Holsteins with a few cases in Shorthorns and Jamaican cattle. Bovine CEP is inherited as an autosomal recessive trait. The condition is present at birth and severely affected calves must be protected from sunlight if they are to survive.

While the predominant clinical manifestations are of teeth, urine discoloration and photosensitization are readily apparent in severely affected animals; these signs vary widely with the degree of the enzyme deficiency, age, time of year, amount of white hair coat, and exposure to sunlight. Marked variations in the urinary

excretion of the porphyrins also occur so that the urine color varies.

Laboratory Detection

Normally, free porphyrins are found in very low concentrations in cells, tissues and body fluids. Thus, the finding of porphyrins in any detectable amounts is always significant. Porphyrin excretion in urine varies, ranging for uroporphyrins between 6.3 and 3,900 µg/dL (0.076–46.96 µmol/L) and coproporphyrins between 2.1 and 8,300 µg/dL (0.032–126.74 µmol/L). At concentrations of 100 µg/dL (1.53 µmol/L) or more, a reddish discoloration is discernible. At 1,000 µg/dL (15.27 µmol/L) or more, an intense red fluorescence of the urine is readily observed when examined with a Wood's light. The principal porphyrins excreted are URO I and COPRO I and the amount of each is also variable.

Bovine fecal porphyrins may be derived from two sources: the bile and from chlorophyll in food. The porphyrins derived from chlorophyll are excluded by the usual analytical method. Essentially, the only porphyrin found in the bile and feces of CEP cattle is COPRO I, and its concentration varies over wide limits. Fecal coproporphyrin varies between 1.9 and 11,800 µg/g (0.003–18.0 µmol/g) and biliary coproporphyrin between 320 and 13,600 µg/dL (4.88–207.67 µmol/L). Only small amounts of COPRO III have been observed in feces.

Only traces of free porphyrins are normally present in the plasma and in the erythrocytes. In bovine CEP plasma variable amounts of URO I (1–27 µg/dL; 0.012–0.33 µmol/L) and COPRO I (4.2–25 µg/dL; 0.064–0.38 µmol/L) are present. A striking difference as compared to the human disease is the high level of free PROTO IX in the erythrocytes of the CEP cow. Excess PROTO IX is commonly found in iron deficiency, hemolytic anemia and in lead poisoning.

The hematologic picture in CEP is one of a responsive hemolytic anemia, with the degree of response directly related to the severity of the anemia. The anemia

in mild cases of bovine CEP is normocytic and in the more severe cases is macrocytic accompanied by reticulocytosis, polychromasia, anisocytosis, basophilic stippling and an increase in nucleated RBC. There is a markedly decreased myeloid:erythroid (M:E) ratio in the bone marrow indicating a marked erythroid hyperplasia.

The presence of porphyrins in the nucleated RBCs is clearly evident by examination of unfixed and unstained bone marrow smears with a fluorescent microscope. These fluorescent RBCs have been called fluorocytes. The fluorescence is seen only in morphologically abnormal nucleated RBCs which contain abnormal nuclear inclusions.

The hematology of newborn CEP calves is strikingly different from that of older CEP calves and cows.¹¹ There is an intense erythrocytic response in the neonatal CEP calf that persists for the first 3 weeks of life. Nucleated RBC counts during the first 24 hours of life range from 5,000 to 63,500/ μ L. Reticulocyte counts are lower than expected (6.4%) and increase to a peak of only 12.5% at 4 days of life.¹¹ Survival time of these reticulocytes in the peripheral circulation is extended beyond their normal one day.

Erythrocyte lifespan is shortened in bovine and in human CEP, which is inversely correlated with RBC coproporphyrin concentration.¹³ The shortest RBC survival time of 27 days (normal = 150 days) was associated with the highest RBC coproporphyrin concentration. The porphyrins through their lipid solubility are presumed to damage the RBC membrane, leading to the hemolysis. Upon exposure of surface capillaries to sunlight, photohemolysis further aggravates the hemolysis. In vivo ⁵⁹Fe metabolic studies were compatible with a hemolytic type of anemia and ineffective erythropoiesis.¹² Plasma iron turnover and transfer rates, RBC iron uptake and organ uptakes were increased as expected in a hemolytic process.

The biochemical defect in the bovine CEP reticulocyte *in vitro* is expressed as an increase in porphyrin synthesis, a marked decrease in heme synthesis, and a delay in the maturation time of reticulocytes.²⁴ The $T_{1/2}$ for the maturation of the reticulocyte is 50 hours compared to a normal value of 3–10 hours. This delay in reticulocyte maturation is thought to be the direct result of the defect in heme synthesis since the rate of heme synthesis controls the rate of maturation of the reticulocyte.

A similar delay in the maturation of the metarubricyte to the reticulocyte was observed in the bone marrow cells of CEP cows²⁰ but there was no effect on earlier nucleated erythroid cells. Ultimately, the accumulation of porphyrins in these cells, whether in bone marrow or in blood, induces delayed maturation or hemolysis.

Detection of the Carrier State

The activity of UROgenIII-Cosyn is considerably less in homozygous CEP cattle as compared to normals¹⁴ and the heterozygotes have UROgenIII-Cosyn activities

intermediate between porphyrics and normals.¹⁹ Similarly low UROgenIII-Cosyn activity is found in human CEP. The genetic aspects of all forms of hereditary porphyrias have been reviewed.¹⁸

Normally in heme synthesis, the combined action of UROgenI-Syn and UROgenIII-Cosyn catalyzes the formation of the normal type III porphyrin isomer, UROgenIII, which ultimately leads to heme formation. In a deficiency of UROgenIII-Cosyn, the type I isomers, UROgenI and COPROgenI are formed, the amounts of which are governed by the relative activities of these enzymes. The type I isomers which are formed cannot be converted into a PROTOgenI so there is no type I heme. This is because COPROgenIII-Ox is specific only for the type III isomer and there is no specific coproporphyrinogen I oxidase. The UROgenI and COPROgenI isomers are readily oxidized to their corresponding free and toxic uroporphyrins and coproporphyrins. These oxidized free porphyrins accumulate in the erythropoietic tissues, developing erythroid cells and in mature RBCs where they induce the hemolysis characteristic of CEP.

Total deficiency of UROgenIII-Cosyn is obviously incompatible with life so that surviving cases of CEP have only a partial deficiency of UROgenIII-Cosyn. The central theme for the metabolic basis for bovine CEP is the genetically controlled deficiency of UROgenIII-Cosyn with the accumulation of the resultant type I porphyrins. These type I porphyrins are the ultimate cause of the clinical and pathophysiological manifestations of CEP.

Bovine Erythropoietic Protoporphyrinuria

Erythropoietic protoporphyria (EPP) is well recognized in humans in which it is inherited as a dominant autosomal trait.¹⁸ Patients do not have the major signs of CEP such as anemia, porphyrinuria or discolored teeth. Photosensitivity of the skin is the only significant clinical manifestation of the disease and this is associated with a high plasma protoporphyrin concentration. The most striking laboratory finding is the high concentration of PROTO IX in the erythrocytes and feces.

In cattle, EPP has a pattern of recessive inheritance and may be sex-linked because to date it has only been documented in females. The photosensitivity also seems to diminish in adult animals. Affected cattle do not have anemia, porphyrinuria, or discoloration of the teeth. RBC and fecal protoporphyrins are very high in comparison to normal cows.²¹ The fundamental enzymatic defect in bovine EPP is a deficiency of FER-Ch with a resulting accumulation of PROTO IX. Low FER-Ch was found in all tissues of EPP calves so that the defect is a total body defect.

Porphyria of Swine

Porphyria in swine is inherited as a dominant trait. Except for the very severe cases, there appears to be little or no effect upon the general health of the pig. Photosensitivity is not seen even in white pigs. The

predominant feature in affected pigs is a characteristic reddish discoloration of teeth that fluoresces upon exposure to UV light. Porphyrin deposition in teeth of newborn pigs is virtually pathognomonic of porphyria in swine. The porphyrins are principally URO I.

The urine of affected pigs is discolored only in more severely affected pigs. The 24 hour urinary excretion of uroporphyrins ranges between 100 and 10,000 μg and for coproporphyrin, is only 50 μg . PBG is absent in the urine. Close similarities in this pattern of porphyrin excretion to that found in bovine CEP are apparent but the localization of the defect in the erythropoietic tissue has not been established. This disease has not been seen in pigs since its original description.

Porphyria of Cats

The first cases of feline porphyria had the typical discoloration of teeth that fluoresced under UV light. Their urine was also discolored and qualitatively positive for uroporphyrin, coproporphyrin, and porphobilinogen. However, there was no evidence of anemia or photosensitization. The porphyria appeared to be inherited as a simple Mendelian autosomal dominant trait analogous to that seen in swine.

Excessive accumulation of URO I, COPRO I, and PROTO IX was observed in the RBCs, urine, feces, and tissues in a family of Siamese cats.⁸ These cats had photosensitivity, severe anemia, and severe renal disease. The principal defect in these cats appeared to be a deficiency of UROgenIII-Cosyn similar to CEP of humans and cows.

Normal Porphyrins

All fox squirrels (*Sciurus niger*) have red bones and this is due to the accumulation of URO I and COPRO I.⁵ Fox squirrel porphyria resembles CEP of humans, cows, and cats by having a deficiency of UROgenIII-Cosyn and accumulation of type I porphyrins in their urine and feces, discolored bones, teeth, and tissues which fluoresce upon exposure to UV light. There is increased erythropoiesis but no apparent hemolytic anemia, no photosensitivity or any other deleterious effects. These relatively benign effects are most likely due to their thick hair coats and nocturnal living habits. It is interesting that an enzyme deficiency with serious health effects in other animals should have evolved as a "normal" characteristic in the fox squirrel. This is understandable when one appreciates that CEP cattle that are always kept indoors and protected from sunlight, thrive and reproduce normally.

The UROgenIII-Cosyn deficiency is found only in the fox squirrel and not in the closely related gray squirrel (*Sciurus carolinensis*). Urine porphyrin excretion in the fox squirrel is 10-fold greater than in the gray squirrel and is further increased when erythropoiesis is stimulated by bleeding. The UROgenIII-Cosyn of fox squirrel erythrocytes is very heat sensitive and this may indicate that its CEP is additionally due to an increased lability of the enzyme.

In the feathers of certain brightly colored birds, e.g. Touracos, and in certain lower animals and microorganisms, porphyrins accumulate but these appear to be normal phenomena.

HEPATIC PORPHYRIAS

This group of porphyrias has been seen only in humans in whom they are the most common group of porphyrias (Table 27.2). The predominant site of the metabolic defect is in the liver and the group is further subdivided on the basis of their principal clinical manifestations. Specific enzyme deficiencies have been identified for all forms of hepatic porphyrias.

δ -Amino-levulinic Acid Dehydratase Porphyria

This rare hepatic form of porphyria has a marked deficiency of the enzyme ALA-D in the homozygous state³ and is called ALA-D porphyria (ADP). It is inherited as an autosomal recessive trait and is characterized by neurologic symptoms without skin photosensitivity.

Acute Intermittent Porphyria

Acute intermittent porphyria (AIP) is the major autosomal dominant form of hepatic porphyria seen in humans and is characterized by acute abdominal attacks and neurological symptoms. Photosensitivity is not a feature of this form. Most patients are not clinically affected unless some form of aggravating factor is present. Attacks are precipitated by several causative factors including barbiturates, sulfonamides, estrogens, and alcohol. The principal urinary finding is the excretion of large amounts of ALA and PBG. The deficient enzyme in this porphyria is UROgenI-Syn.

Porphyria Cutanea Tarda

Porphyria cutanea tarda (PCT) is caused by a deficiency of UROgen-D and presents as both a sporadic form and a familial form. The sporadic form is acquired and the familial form is inherited as an autosomal dominant. The characteristic clinical signs of PCT are the photosensitivity. The disease occurs in mid to late adult life and common precipitating causes of this disease are alcohol and estrogens. The disease can be successfully treated by avoidance of these substances. There is a decrease in hepatic UROgen-D in both forms but the enzyme deficiency is found in extra-hepatic tissues only in the familial form¹⁷. In the sporadic form, RBC UROgen-D activity is normal whereas in the familial form, RBC UROgen-D is less than 50% of normal.¹⁵ Sporadic PCT has a significant increase in GGT whereas the familial form has normal GGT activity.¹⁸ Furthermore, the ratio of fecal COPRO III:COPRO I when combined with the plasma GGT was found to give an even more accurate differentiation of the sporadic form from the familial form.²

Hepatoerythropoietic Porphyria

Hepatoerythropoietic porphyria (HEP) is a form that clinically resembles CEP but there is a marked deficiency of UROgen-D as in PCT. It is thought to be the homozygous form of familial PCT. HEP is characterized by a very severe photosensitivity but there is no liver involvement.

Harderoporphyria

This is a rare form of porphyria in which the propionate group on the A ring only is converted to a vinyl group. The normal next step of B ring conversion is somehow disrupted. There is a deficiency of COPROgenIII-Ox but the mechanism explaining why the groups on both rings are not oxidized is unknown.

Hereditary Coproporphyria

Hereditary coproporphyria (HCP) is clinically similar to PCT with a mild cutaneous photosensitivity and it may also have neurologic symptoms as in AIP. Like AIP, HCP is commonly precipitated by drugs and alcohol. As in harderoporphyria, COPROgenIII-Ox is the deficient enzyme.

Variegate Porphyria

The symptoms of variegate porphyria (VP) are generally more variable than the other forms but in most cases, acute abdominal attacks and photosensitivity are seen. Variegate porphyria is most common among the South African white population and is inherited as an autosomal dominant. There is a deficiency of PROTOgenIII-Ox, which can be observed in cultured fibroblasts and in leukocytes of VP patients.⁹

ACQUIRED TOXIC PORPHYRIAS: THE PORPHYRINURIAS

Chemical Porphyrias

The two major forms of the acquired toxic porphyrias are those due to organic chemical intoxication and to heavy metal poisonings, mainly lead. Experimentally, hexachlorobenzene (HCB), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), allylisopropylacetylcarbamide (Sedormid), or dihydrocollidine (DHC) have been used to produce the hepatic forms of porphyria.

Lead Poisoning

Lead poisoning occurs in all domestic animals and is a significant clinical problem, particularly in the dog and in children. The principal clinical features are related to the gastrointestinal and nervous systems (see Chapter 17). Anemia is usually seen only in long standing chronic lead toxicities. The anemia has certain features that are suggestive of lead poisoning but these are not

diagnostic. A non-responsive mild to moderate normocytic normochromic anemia with basophilic stippling and nucleated RBCs (NRBCs) out of proportion to the degree of anemia may be seen. More than 15 stippled cells per 10,000 RBCs is suggestive of lead poisoning in dogs and >40 stippled cells per 10,000 erythrocytes is reported to be diagnostic.²⁵ Stippling is thought to represent aggregates of ribosomal RNA that have not been degraded to their nucleotides and subsequently dephosphorylated by pyrimidine-5'-nucleotidase (5'NT). Lead has been shown to decrease the activity of 5'NT in humans²⁶ and in calves.⁷

Lead is known to have widespread toxic effects on sulfhydryl-, carboxyl-, and imidazole-containing proteins that would include enzymes, cell proteins, globins, and membrane proteins. However, only a few are altered in a way that is of diagnostic value. Hemoglobin synthesis is disrupted and this is the major mechanism of the anemia of lead poisoning. The anemia, however, occurs late in chronic lead poisoning and its non-specific nature makes it of less diagnostic importance than is usually attributed to it.

Several of the enzyme systems of heme synthesis are very sensitive in early exposure to small quantities of lead. The most sensitive are ALA-D and FER-Ch, and these enzymes and their accumulated substrates are widely used as screening tests for lead exposure. Erythrocyte ALA-D is strongly inhibited by lead and as a result, ALA increases in plasma and is excreted in the urine. Measurement of ALA is difficult and the result lacks sensitivity for low-level lead exposure. Therefore, erythrocyte ALA-D is more commonly assayed. The ratio of ALA-D activity assayed at two different pH values demonstrates ALA-D inhibition at low blood lead concentrations of 10–15 µg/dL (0.50–0.70 µmol/L). This is thought to be a more sensitive and reliable index of the blood lead concentration.⁴

FER-Ch is the second major enzyme that is strongly inhibited by lead and as a result, PROTO IX accumulates in the RBCs. RBC ZnPROTO IX increases when blood lead concentrations are at 15–18 µg/dL (0.75–0.85 µmol/L). This is well below the blood lead concentration of >30 µg/dL (1.45 µmol/L) that is currently regarded as diagnostic for lead poisoning in children. Marked elevations in RBC PROTO IX are also found in experimental lead poisoning in calves.⁶ Modern fluorometers specifically designed to measure porphyrins have greatly simplified the assay. For these reasons, the current test of choice to monitor lead exposure is the blood ZnPROTO IX concentration.

The final diagnosis of lead poisoning ultimately rests upon the measurement of blood lead concentration and this is best done using atomic absorption spectrophotometry. It is clear that the heme synthetic pathway is affected by blood lead concentrations well below those that are considered normal. The reported reference interval for blood lead in the dog is 10–50 µg/dL (0.48–2.41 µmol/L) and a blood lead concentration of >60 µg/dL (2.90 µmol/L) is diagnostic of lead poisoning.²⁵ In the light of current knowledge, a blood level of 30 µg/dL (1.45 µmol/L) should be considered diagnostic of

lead poisoning in the dog as well as in all veterinary species.

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Hereditary Erythrocyte Enzyme Abnormalities

URS GIGER

Phosphofructokinase Deficiency
Canine Phosphofructokinase Deficiency

Pyruvate Kinase Deficiency
Canine Pyruvate Kinase Deficiency
Feline Pyruvate Kinase Deficiency

Acronyms and Abbreviations

ADP, adenosine diphosphate; ATP, adenosine triphosphate; BFU-E, burst forming units-erythroid; CFU-E, colony forming unit-erythroid; DNA, deoxyribonucleic acid; DPG, diphosphoglycerate; EDTA, ethylenediaminetetraacetic acid; G6PD, glucose-6-phosphate dehydrogenase; L-M-P-PFK, liver, muscle, platelet phosphofructokinase; L-M1/M2-R-PK, liver, muscle, red blood cell pyruvate kinase; PFK, phosphofructokinase; PK, pyruvate kinase; RBC, red blood cell.

As the committed erythroid precursor cells proliferate and develop into mature red blood cells (RBCs) in the bone marrow, their cellular metabolism undergoes many changes. While the burst forming units-erythroid (BFU-E) and colony forming unit-erythroid (CFU-E) cells are geared to massively expand the erythroid cell line, which is under the control of erythropoietin, major metabolic and structural shifts occur, particularly during the later erythroid stages, to achieve the highly specialized functions of mature RBCs (see Chapters 6, 20, and 21).⁴⁰ To that end, during rubricyte and metarubricyte (also known as normoblasts) stages until the reticulocyte stage, heme and alpha- and beta-globin syntheses are in full force to produce hemoglobin, reaching one-third of a mature RBC's mass. Heme synthesis occurs in both mitochondria and cytoplasm involving several enzymatic steps. Hereditary enzymatic defects in heme synthesis, also known as porphyrias, cause tissue (bone and teeth) accumulation and urinary as well as fecal excretion of varied porphyrins, and can lead to hematological, neurological, gastrointestinal, and cutaneous signs. Porphyrias and their classification and characterization to the molecular level in cats, pigs, sheep, and cattle are described in Chapter 27. While sickle cell disease and thalassemia are the most common RBC disorders in humans, interestingly, no hemoglobinopathies causing anemia have thus far been documented in domestic animals. However, hereditary methemoglobinemias, either due to a cytochrome b5 (methemoglobin) reductase deficiency or a yet unknown mechanism, have been observed in dogs, cats, and even a Rhesus monkey, and are typically asso-

ciated with cyanosis and erythrocytosis rather than anemia (see Primary erythrocytosis in Chapter 25).^{22,32,34,39} Glucose-6-phosphate dehydrogenase (G6PD) of the hexose-monophosphate shunt is the most common hereditary (and only x-chromosomal) RBC enzyme deficiency in humans (Fig. 28.1); these patients are generally not anemic, but can develop hemolytic crises with oxidative stress (drugs, infections, flava beans [flavism]).² While in a large canine survey only a single Weimeraner dog was found to have a reduced G6PD activity without any hematological abnormalities,⁷¹ an American saddlebred colt with G6PD had anemia with eccentrocytosis.^{39,59} Furthermore, horses with RBC flavin adenine dinucleotide deficiency have both eccentrocytosis (attributable to severe deficiency in glutathione reductase activity) and methemoglobinemia (attributable to cytochrome b5 reductase deficiency); the dual enzyme deficiency occurs because flavin adenine dinucleotide is a required cofactor for both enzymes.²⁹ Although various erythrocytic membrane defects have also been described affecting cytoskeletal proteins, such as spectrin and band 4.1, others remain undefined and may involve enzymatic pumps to maintain electrolyte and calcium homeostasis as suspected for stomatocytosis in the Alsaka Malamute and Miniature and Middle Schnauzer (see Chapter 29).⁷⁶

Devoid of a nucleus and mitochondria the metabolism of the mature mammalian RBC is fairly restricted, and its energy is solely generated by anaerobic glycolysis, also known as the Embden-Meyerhof pathway (Fig. 28.1; see Chapter 21).⁴⁰ Metabolism of one molecule of glucose to two molecules of lactate leads to the net

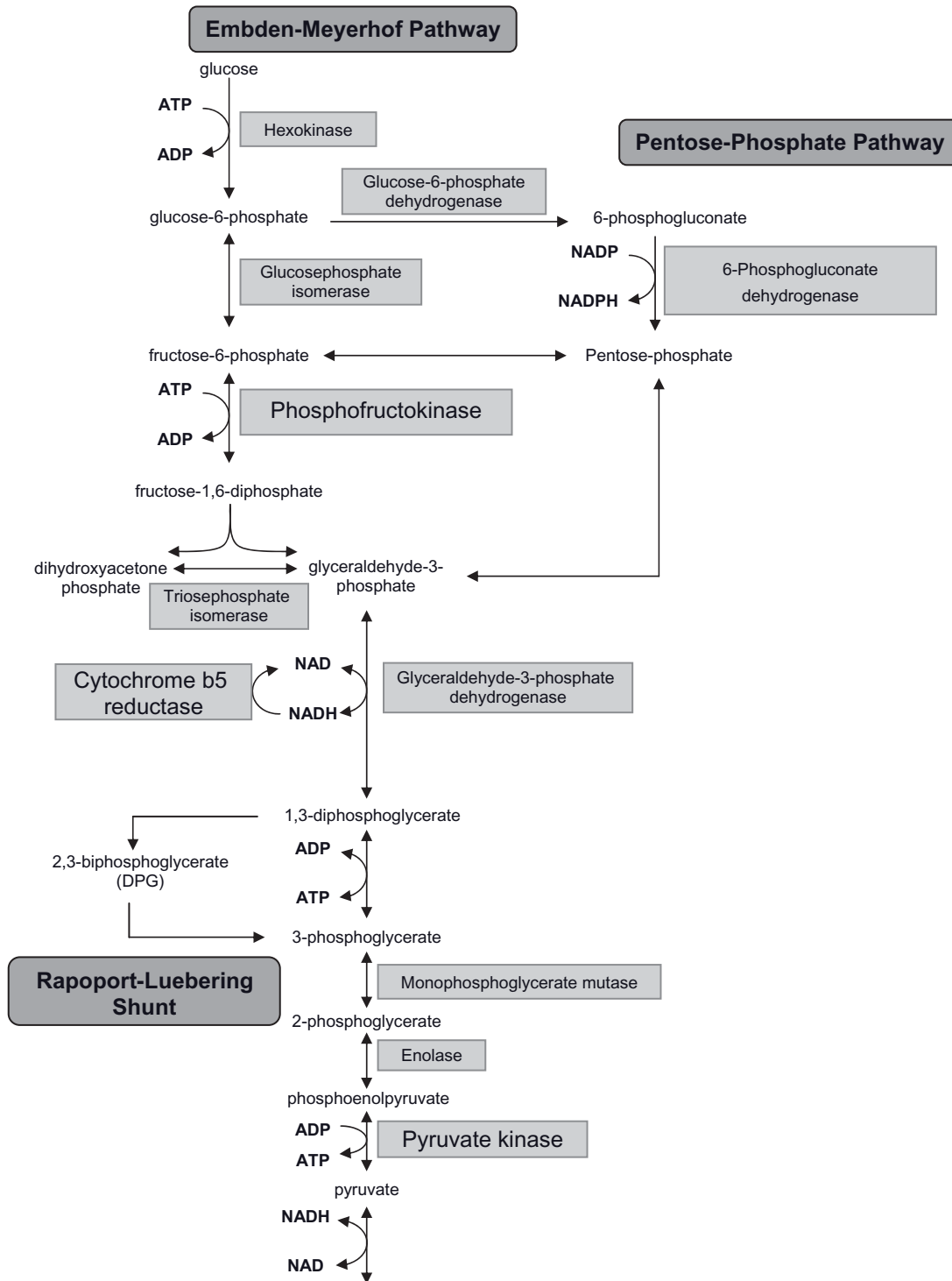


FIGURE 28.1 Embden-Meyerhof (anaerobic glycolytic) pathway with hexose-monophosphate and Rapoport-Luebering shunt in RBCs (simplified). ATP, adenosine triphosphate; ADP, adenosine diphosphate.

production of two molecules of ATP. The rate of glycolysis depends on the need for ATP of RBCs to maintain the shape, deformability, membrane transport, and metabolic functions such as phosphorylation and synthesis of purines, pyrimidines, and glutathione. Over a dozen enzymes are involved in glycolysis, which are

associated with the two ancillary pathways, unique to RBCs, the pentose-phosphate (hexose-monophosphate) pathway and Rapoport-Luebering shunts. Some of these enzymes exist in different isoforms allowing for cell and tissue specific expression and regulation. The key regulatory enzyme of anaerobic glycolysis is

phosphofructokinase (PFK). Whereas ATP is the most important inhibitor of PFK activity, inorganic phosphate, AMP and ADP action is stimulatory. In addition, glucose-1,6-biphosphate and fructose-2,6-biphosphate are activators. Under maximally activating conditions for the enzyme PFK, an enzyme distal in glycolysis, pyruvate kinase (PK), becomes rate-limiting (Fig. 28.1).

It is, therefore not surprising that a deficiency of either PFK or PK activity will lead to RBC malfunction and premature RBC destruction, thereby causing hemolytic anemia. Although both erythroenzymopathies impair the same metabolic pathway, their metabolic and hematological abnormalities and clinical presentations are distinctly different and can vary between species. Subsequently, both erythroenzymopathies will be reviewed separately and contrasting features between the two disorders as well as affected species will be highlighted.

PHOSPHOFRUCTOKINASE DEFICIENCY

Phosphofructokinase (PFK; EC 2.7.1.11) catalyzes the regulatory phosphorylation step of fructose-6-phosphate to fructose-1,6-biphosphate. There are three isoforms of PFK, referred to as muscle (M-PFK), liver (L-PFK), and platelet (P-PFK) that are encoded by three different genes.⁷⁸ The active PFK enzyme is a homo- or heterotetramer composed of one or more isoforms; each of these combinations provides unique enzyme kinetic properties. Their expression is cell and tissue specific and developmentally regulated. Skeletal muscle contains exclusively M-PFK homotetramers.⁵⁴ Human RBCs express equal amounts of M- and L-PFK, whereas in canine RBCs, the M-PFK isoform predominates in a ratio of 86:14 over P-PFK.⁷⁸ Furthermore, during myogenesis⁷ and erythropoiesis as well as postnatal development,³⁰ the isoform composition in these cells changes from L-PFK to M- and P-PFK. Deficiency of M-PFK has been associated with hemolysis and myopathy in humans and dogs, whereas in horses and Holstein calves PFK deficiency apparently did not cause any hematologic abnormalities⁷⁵ and will, therefore, not be discussed here.

Muscle-type PFK deficiency, also known as Tarui-Layzer syndrome, is a rare genetic disorder in humans characterized by metabolic myopathy and a well-compensated hemolytic disorder.^{25,62} PFK-deficient humans exhibit exercise intolerance, muscle weakness, and muscle cramping on exertion. Because of the residual half-normal PFK activity in affected RBCs, contributed by L-PFK isozymes and lack of alkaline fragility of human RBCs, overt clinical signs of hemolysis occur rarely. In fact, human patients can over-compensate their hemolytic component, and develop a mild erythrocytosis, because PFK-deficient erythrocytes do not readily release oxygen from hemoglobin. The ensuing tissue hypoxia accelerates erythropoiesis. Several mutations in the M-PFK gene have been described in human patients.⁵⁵

Canine Phosphofructokinase Deficiency

Canine M-PFK deficiency was first described as an autosomal recessive trait in English Springer Spaniels in 1985 and was the first defect characterized at the molecular level for a common inborn error in dogs.^{9,10,12,13,23,68} PFK-deficient dogs have a nonsense mutation in the last exon of the M-PFK gene.^{69,70} The resulting change from a tryptophan codon to a stop codon causes truncation of the M-PFK protein by 40 amino acids, leading to rapid degradation and complete deficiency of the M-PFK enzyme.^{53,70} Affected dogs completely lack PFK activity in muscle and have 8–22% of control PFK activity in RBCs due to residual P- and L-PFK expression (other tissues appear less affected as they express higher levels of other PFK isoforms).^{15,30,33,36,78} The metabolic block at the PFK step results in deficiency of ATP and DPG in RBCs, reduced blood lactate production, and accumulation of sugar phosphates and glycogen in muscle; hence also the term glycogenosis or glycogen storage disease type VII.^{10,11,15} This metabolic pattern is also known as a metabolic crossover, indicating the specific metabolic block in a pathway.

In erythroenzymopathies, the mechanism of accelerated lysis is generally not known, but it is assumed to be caused by energy depletion. In dogs, the compensated hemolytic disorder of PFK deficiency is accentuated by life-threatening hemolytic crises. A unique mechanism has been documented to be responsible for intravascular lysis, namely an increased alkaline-induced hemolysis.⁹ Although incompletely understood and possibly related to an aberrant RBC calcium homeostasis, canine RBCs are more alkaline fragile than cells from other species.^{41,79} RBC calcium homeostasis is disturbed in PFK deficiency, which results in an energy imbalance that accelerates adenine nucleotide pool depletion. Ca²⁺-calmodulin activation of RBC AMP deaminase contributes to this metabolic dysregulation and limits ATP, thereby likely contributing to increased RBC lysis.⁶³ In vivo, the intracellular pH of RBCs is determined by organic phosphate and chloride anions. Because PFK-deficient RBCs have markedly decreased DPG concentrations, chloride ions move in and increase RBC pH.^{9,11,29} Thus, PFK-deficient RBCs start lysing at about pH 7.4 (Fig. 28.2A). Consequently, even minor systemic alkalemia or hyperthermia can induce intravascular hemolysis. Alkalemia is associated with any form of hyperventilation.^{9,13}

The low DPG content of PFK-deficient RBCs markedly increases oxygen affinity and this is reflected in a left shift of the hemoglobin-oxygen dissociation curve.^{9,10} The relative tissue hypoxia impairs, for instance, muscle and other tissue metabolism and stimulates renal erythropoietin synthesis and erythropoiesis. Therefore, aside from the occasional hemolytic crises associated with anemia, the chronic hemolytic disorder can be fully compensated with a robust regenerative response even at normal hematocrit. However, erythrocytosis as seen in PFK-deficient humans does not occur in affected dogs.

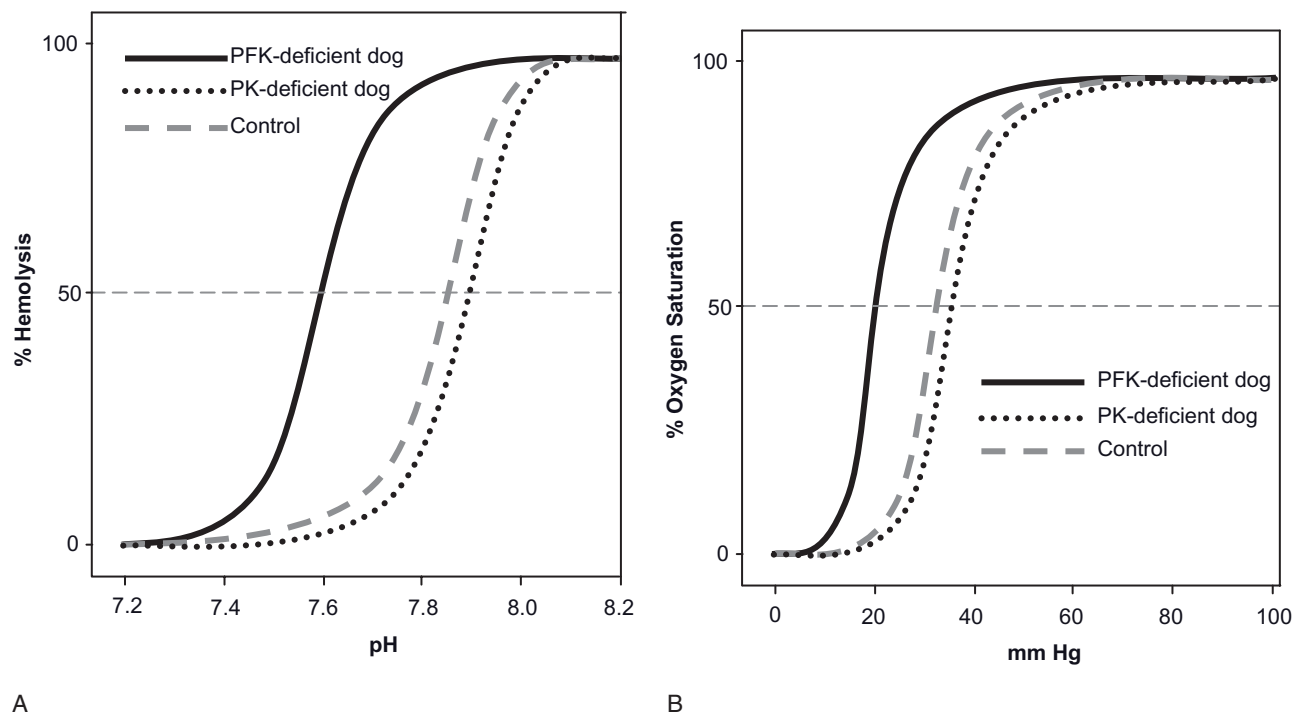


FIGURE 28.2 (A) Alkaline fragility of RBCs from a phosphofructokinase (PFK)-deficient English Springer Spaniel and pyruvate kinase (PK)-deficient Beagle compared to a healthy control dog. RBCs were incubated at various pH values for 1 hour at 37°C 24 hours after collecting the blood samples. Note that RBCs from PFK-deficient dog are very alkaline fragile, while those from PK-deficient dogs behave like controls. (B) Hemoglobin-oxygen dissociation curve of RBCs from phosphofructokinase (PFK)-deficient English Springer Spaniel and pyruvate kinase (PK)-deficient Beagle. Note the severe left shift of the curve of the PFK-deficient cells (higher hemoglobin-oxygen affinity, $P_{50} = 17$ mmHg), while pyruvate kinase (PK)-deficient canine RBCs are slightly right-shifted (lower hemoglobin-oxygen affinity, $P_{50} = 32$ mmHg) compared to canine control blood (28 mmHg).

Sporadic intravascular hemolytic crises associated with pigmenturia, anemia, or jaundice are the hematological hallmark findings of PFK deficiency in dogs.^{9,13,35} These episodes are induced by hyperventilation-associated events such as excessive panting and barking, strenuous exercise and high environmental temperatures. Thus, guests or a new pet at the patient's home, a visit to the veterinary clinic, boarding, and training for field trialing can precipitate hemolysis. These crises can be first observed at a few months of age, although some animals may not experience any problems until several years of age, or they may be completely missed until the owner looks for them. During these episodes, which generally last one to several days, affected animals can have pale to severely icteric mucous membranes, are lethargic, inappetent, and often febrile, and splenomegaly may be noted. Thus, PFK deficiency can readily be confused with immune-mediated and other acquired hemolytic anemias. Pigmenturia is characterized by hemoglobinuria during crises, whereas hyperbilirubinuria is persistently strong. The anemia may become life-threatening with hematocrits as low as 5%, but is always macrocytic, hypochromic and strongly regenerative with corrected and absolute reticulocyte counts of 4–25% and 200,000–1,200,000/ μ L, respectively. The apparent half-life of chromium-labeled PFK-deficient RBCs is 16 days compared to 20–28 days for

normal canine cells. Beside polychromasia, marked anisocytosis and normoblastosis (but no poikilocytosis) are observed on microscopic examination of a blood smear. Yet unexplained, leukocytosis and hyperglobulinemia also may be present. The plasma may be discolored because of hemoglobinemia and bilirubinemia. Transient hyperkalemia is due to lysis of high potassium-containing reticulocytes and young RBCs.^{9,13}

The debilitating exertional metabolic myopathy typically seen in human PFK-deficient patients is less commonly observed and frequently milder in affected dogs. PFK-deficient dogs may develop muscle signs on exertion, even only for a short distance.^{3,14,15,51,52} In fact, muscle cramping in one of the limbs even after mild exercise is the most dramatic presentation.¹⁴ Affected dogs may suddenly refuse to run and have high serum creatine kinase activity. Despite a complete lack of PFK activity in muscle, clinical signs of myopathy in deficient dogs are generally mild and rare, presumably due to the high oxidative rate of canine skeletal muscle.⁷² Nevertheless, major impairments in oxidative and anaerobic muscle metabolism have been demonstrated experimentally.^{3,51,52} Aside from the acute signs of cramping, affected dogs may only show clinically mild exercise intolerance and intermittent muscle wasting. The serum creatine kinase activity is normal to slightly increased. PFK-deficient skeletal muscle accumulates

slightly more glycogen and occasionally amylopectin, an abnormal glycogen.^{14,31} Interestingly a couple of Whippets with PFK deficiency also showed clinical signs of a cardiomyopathy probably resulting from the M-PFK isozyme deficiency in heart muscle.⁸

PFK deficiency is an autosomal recessive trait¹⁰ and occurs commonly in the English Springer Spaniel breed.^{20,44} Since the first description in 1985,⁹ over 200 cases have been documented, but the gene frequency remains unknown. A randomized survey of American Kennel Club (AKC)-registered championed and bred English Springer Spaniels in 1998 revealed that 4% of field trial and and 2.7% of show (bench) English Springer Spaniels were still carriers. Based on extensive pedigree analyses, a common ancestor goes at least back to the 1960s, if not earlier. PFK deficiency also has been reported in American Cocker Spaniels,¹⁹ and several mixed breed dogs and Whippets.⁸ As they all had the same disease-causing DNA mutation, these animals must have a common ancestor.

PFK deficiency may be suspected based upon signalment, family history, typical clinical signs, and suggestive laboratory test abnormalities. While the pigmenturia is generally marked (i.e. orange to black), it may not be noted by the owners, particularly with a female dog, and other signs may be mild unless the dog is rigorously exercised. A definitive diagnosis requires proof of the PFK mutation or reduced RBC PFK enzyme activity. A simple polymerase-chain reaction (PCR)-based DNA test accurately identifies PFK-deficient dogs (homozygotes, affected; two mutant alleles) and carriers (heterozygotes; one mutant and one normal allele) in the English Springer Spaniel, American Cocker Spaniel, and Whippet as well as the mixed breeds.^{8,18,19,70} EDTA-anticoagulated blood, but also a drop of blood dried on special filter paper (Guthry paper), a buccal swab with a cytobrush, and even semen from a semen bank or formalized tissues can be suitable sources for DNA extraction and PCR testing. Whereas the PCR test and differentiation either by sequencing or restriction-enzyme digest are simple and permit a diagnosis of deficient, carrier, and normal animals from the first day of life, the PFK enzyme activity test is cumbersome and not accurate until two months of age. Normal and affected neonatal dogs express large quantities of L-PFK in RBCs, which increases the overall PFK activity.³⁰ As the PCR-based test is mutation-specific, the measurement of PFK enzyme activity may, however, be indicated in other breeds and species, where the same disease is suspected. The PCR-based PFK test is recommended for use as a screening test for English Springer Spaniels (1) with suspicious clinical signs, (2) related to affected or carrier dogs, (3) used for breeding, and (4) prior to training for field trialing. In Whippets and American Cocker Spaniels, PFK deficiency seems to be more isolated and thus general screening is not recommended.

PFK-deficient dogs can have a normal life expectancy, if crises-inducing situations are avoided.¹³ Dogs experiencing a hemolytic crisis are provided supportive care including, if needed, type-matched blood transfu-

sions and in case of repeat transfusions cross-matched blood. Fever and excessive panting or barking should be avoided whenever possible. Diamox, a carbonic anhydrase inhibitor, may acidify the blood, and aspirin and dipyron may counter the fever accompanying hemolysis, thereby preventing further intravascular lysis during an acute crisis; however, they have not been proven effective in clinical practice. Similarly, because of disturbed purine metabolism, adenine could be considered as treatment, but efficacy and safety data are lacking.⁶³ Despite massive intravascular hemolysis, hemoprotein-induced acute nephropathy has not been observed. Nevertheless, hemolytic dogs should be well hydrated. Affected dogs recover from these crises within days. The management of the cardiomyopathy seen in PFK deficient Whippets may represent one of the breed-specific complications limiting their life expectancy.⁸

Finally, experimental bone marrow transplantation has successfully corrected the hematological abnormalities and abolished the hemolytic crises. Moreover, the corrected RBCs also normalized the hemoglobin-oxygen affinity and, thereby, positively affected oxygenation of muscle and aerobic muscle function.^{51,52} Because of the morbidity and mortality associated bone marrow transplantations even when a dog leukocyte antigen (DLA)-matched donor (PFK carrier or normal) sibling is available and the relatively mild signs, when preventative measures are instituted, bone marrow transplantation cannot be recommended for this inborn error of metabolism.

PYRUVATE KINASE DEFICIENCY

Pyruvate kinase (PK; EC 2.7.1.40) catalyzes the ATP-generating conversion of phosphoenolpyruvate to pyruvate and is, therefore, an important regulator in the terminal glycolytic pathway. There are two different genes coding by alternative splicing a total of four developmentally dependent and tissue-specific PK isoforms.^{42,50,57,58} The PKLR gene encodes RBC (R-PK) and liver (L-PK) isoenzymes, whereas the PKM gene generates the muscle (M1-PK and M2-PK) isoforms. R-PK is expressed almost exclusively in mature RBCs and has a different first exon making the amino-terminal sequence longer when compared to that of L-PK. In contrast, erythroid precursors express M2-PK and switch to R-PK isoforms during differentiation to mature RBCs.⁵⁰ Erythrocytic PK deficiency causing hemolytic anemia has been described in humans, dogs, cats, and mice.^{43,55,56,73}

Pyruvate kinase deficiency is the most prevalent hereditary non-spherocytic hemolytic anemia caused by a glycolytic enzymopathy.^{55,74} Many mutations in the PKLR gene have been found to cause R-PK deficiency in human patient; some of them are common in certain ethnic (Amish) and geographical regions.^{5,55} Most of the patients, however, are compound heterozygotes with two different mutant alleles. Clinical signs in PK-deficient humans are very variable, ranging from a mild compensated hemolytic disorder to severely anemic

patients who become transfusion-dependent, may develop iron overload (particularly when receiving regular transfusions), and die during early childhood. Splenectomy has been shown to be helpful in some human patients with severe anemia; iron chelation and bone marrow transplantation are other options.^{25,74}

Canine Pyruvate Kinase Deficiency

In 1971, erythrocytic PK deficiency was first recognized in Basenji dogs.⁶⁴ It served as the classic example of an inborn error of metabolism in veterinary medicine, despite the fact that the biochemical derangements turned out to be more complex, making a clinical diagnosis more difficult. R-PK deficiency occurred most frequently in Basenjis,^{17,64,65} but has also been reported in Beagles,^{16,28,60} West Highland White^{4,67} and Cairn Terriers,⁶⁴ Miniature Poodles, Dachshunds,⁴⁵ Chihuahuas, and American Eskimo Toy dogs. In Basenjis, a single base-pair deletion in exon 5 of the R-PK gene has been identified causing a frameshift and severe truncation of the protein.^{81,82} In contrast, a six base insertion ambiguously positioned at the 3'-end of exon 10 in the R-PK sequence was found in West Highland White Terriers.⁶⁷ This insertion results in the in-frame addition of two amino acids, threonine and lysine, and is likely also the causative mutation in Cairn Terriers,⁶³ a related breed. In the Beagle, an exon 1 mutation has been found while in the other breeds a disease-causing mutation has yet to be confirmed. Despite the varied mutations in R-PK, the biochemical changes and clinicopathologic manifestations of PK deficiency in any of the reported canine breeds appear identical.

The R-PK deficiency in RBCs of affected dogs can be demonstrated by agarose gel electrophoresis, immunoblotting, and, immunoprecipitation studies.^{17,37,38} However, all affected dogs express M2-PK in RBCs, which increases total RBC PK activity of affected dogs, and, therefore, is misleading. For instance, the Miniature Poodles with non-spherocytic anemia and osteosclerosis may well have had R-PK deficiency,⁶¹ despite high RBC PK activity *in vitro*. In contrast M2-PK expression is rarely observed in human patients. This M2-PK isozyme, which is normally present in erythroid precursor cells, appears to be heat-labile and dysfunctional *in vivo*, because affected RBCs show a severe metabolic block at the PK step.¹⁷ Beside the accumulation of proximal glycolytic metabolites in RBCs, the DPG content is also increased. The resulting right shift of the hemoglobin-oxygen dissociation curve allows for improved oxygen delivery, thereby ameliorating the clinical signs of severe anemia in PK-deficient dogs.

PK-deficient dogs frequently present for evaluation at a few months to a couple of years of age because of intermittent weakness and exercise intolerance. They appear to adapt very well to the anemia, presumably due to the favorable tissue oxygenation. The hematocrit ranges between 12% and 28%; the anemia is highly regenerative with reticulocyte counts of 10–90%. Echinocytes have been documented.⁶⁴ In contrast, canine PK-deficient RBCs have normal alkaline fragility

and readily release oxygen compared to canine PFK-deficient RBCs (Fig. 28.2). The erythrocyte half-life is only 5.8 days compared to 19–28 days in normal dogs.¹⁷ Hepatosplenomegaly, due to extramedullary erythropoiesis and hemosiderin deposition, may also be noted. Affected dogs die because of anemia or hepatic failure between 1 and 9 years of age, with Basenjis having a more severe form than Beagles and West Highland White Terriers. Interestingly, all PK-deficient dogs develop a progressive myelofibrosis and osteosclerosis that remain unexplained and do not occur in other species with PK deficiency.^{4,16,17,28,45,63,64,66} The osteosclerosis may be identified by radiography of long bones at 1 year of age and completely obstructs the marrow cavities by 3 years in Basenjis, but occurs later in other breeds.

A diagnosis of PK deficiency is strongly suggested by the concurrent occurrence of a highly regenerative severe anemia and osteosclerosis in a dog. Generally, routine screening tests rule out or are not supportive of acquired anemias and the anemia is persistent and appears well-tolerated. PCR-based PK tests are available for the Basenji,^{80,81} Beagles, and West Highland White Terrier breeds.⁶⁷ These DNA tests are accurate to detect affected and carrier dogs in these breeds. However, because they are mutation-specific, they cannot be used for other breeds. In other breeds, cumbersome PK enzyme activity assays using agarose gel electrophoresis, immunologic, and heat-lability methods specific for R-PK are required due to M-PK expression. PK carriers do not express M-PK and, therefore, have intermediate (~50% of normal) RBC PK activity. Unfortunately, the PK activity assays are not very accurate in detecting carriers, as the carrier and normal ranges overlap.¹⁷ PK deficiency has most commonly been reported in the Basenji, West Highland White Terrier, and Beagle breeds, but the PK mutation frequency remains unknown in all canine breeds.

Affected dogs are managed symptomatically in clinical practice. Because of the excellent DPG-facilitated oxygen release from hemoglobin, PK-deficient dogs adapt well despite severe anemia. They rarely need transfusions except for their terminal stage. Bone marrow transplantation, performed experimentally,⁸⁰ corrects the hemolytic anemia and halts the osteosclerosis and hemosiderin deposition, whereas splenectomy has not been shown to be effective in slowing the hemolysis and anemia.

Feline Pyruvate Kinase Deficiency

In the early 1990s, erythrocytic pyruvate kinase deficiency was identified and characterized in the Abyssinian, Somali and Domestic Shorthair cats.^{6,21} A 13 base-pair deletion at the 3'-end of exon 5 in the R-PK cDNA, but not the genomic sequence is caused by a splicing defect and results in a severe reduction in RBC PK enzyme activity. In cats there is no anomalous M2-PK expression in RBCs and no osteosclerosis, which are observed in all PK-deficient dogs. Since the original observations of the PK-deficient Abyssinian

and Somali cats, over 3,000 have been screened; approximately 12% are affected and 24% are carriers, making this one of the most frequent causes of anemia in these breeds. Since then, PK-deficient Abyssinian and Somali cats have been reported from Europe^{26,27,47,48,77} and Australia.^{1,49} In addition, a few Domestic Shorthair cats have also been found with the same mutation in the United States. Interestingly, a specific pathogen-free research colony of domestic cats also carries this same PK mutation and is responsible for an unexplained mild to moderate intermittent anemia.

Affected cats have chronic intermittent hemolytic anemia that might not first be recognized until advanced age. The anemia is intermittent, mild to severe with PCV ranging from 5 to 35%. It is slightly macrocytic-hypochromic and slightly to strongly regenerative (reticulocyte count 45,000–290,000/ μL).^{24,47,48} Moreover, PK-deficient cats frequently have a marked lymphocytosis and hyperglobulinemia. Many of these features are similar to cats with increased RBC osmotic fragility with splenomegaly in the same breeds.⁴⁶ However, PK-deficient cats typically have a greater regenerative response and only mild splenomegaly and mild osmotic fragility (Fig. 28.3) Extrahepatic biliary obstruction due to bilirubin calculi is seen in affected cats.^{8,27} It should be noted that many deficient cats remain completely asymptomatic for years and the associated anemia may not be the primary reason for a PK-deficient cat's illness.

PK-deficient cats frequently do not require treatment, just like PK- and PFK-deficient dogs. However,

during a crisis, deficient cats may need to be transfused with AB blood-type compatible blood. In case of severe splenomegaly or persistently severe anemia, splenectomy appears to reduce the severity of the hemolytic crises.²⁴

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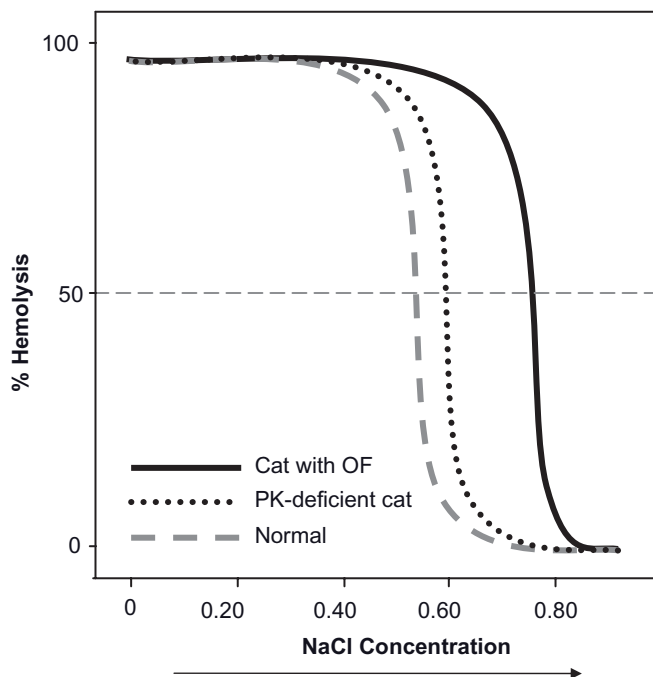


FIGURE 28.3 Erythrocyte osmotic fragility (OF) curve from a PK-deficient Abyssinian cat and an Abyssinian with increased OF and splenomegaly. RBCs were exposed in vitro to different saline concentrations from 0% (water) to 0.85% (physiological saline) at room temperature for 30 min.

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Erythrocyte Membrane Defects

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Hemolytic Anemias Caused by Hereditary Red Cell Membrane Defects

Hereditary Spherocytosis

Hereditary Band 3 Deficiency in Cattle
(Band 3^{Bov.Yamagata})

Spectrin and Ankyrin Deficiencies in Animals

Hereditary Elliptocytosis

Hereditary Protein 4.1 Deficiency in Dogs

Spectrin Anomaly with Elliptocytosis in a Dog

Hereditary Stomatocytosis

Hereditary Stomatocytosis in Dogs and Cats

HSt in Miniature and Standard Schnauzers

HSt in chondrodysplastic Alaskan Malamute dwarf dogs

Familial stomatocytosis-hypertrophic gastritis in dogs

Hemolytic anemia with increased erythrocyte osmotic fragility in cats

Diagnostic Approaches to Erythrocyte Membrane Defects

Membrane Transport Defects

Amino Acid Transport Deficiency in Animals

High Membrane Na,K-ATPase Activity in Dogs

Acronyms and Abbreviations

DHS, dehydrated hereditary stomatocytosis; HE, hereditary elliptocytosis; HPP, hereditary pyropoikilocytosis; HS, hereditary spherocytosis; HSt, hereditary stomatocytosis; OHS, overhydrated hereditary stomatocytosis; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; PCR-SSCP, PCR-single strand conformation polymorphism; RBC, red blood cell; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Red blood cells (RBCs) must be very durable and flexible to undergo marked deformation under high shear stress condition so that they can survive during repeated passages through the microcirculation. These important properties are determined by three major elements of their membranes: a lipid bilayer, integral or transmembrane proteins, and a membrane skeletal network (Fig. 29.1). A lipid bilayer provides a permeability barrier between the cytosol and the external environment. Major constituents of the membrane skeleton mechanically support the plasma membrane and are organized into a lattice-like meshwork that is linked with both integral membrane components and cytoskeletal elements. Some of the transmembrane proteins embedded within the lipid bilayer ensure selective permeability to maintain RBC homeostasis. Accelerated destruction of erythrocytes (hemolytic anemia) may occur when these properties of the RBC membrane are affected or deficient by some genetic defects. This chapter reviews the current progress in the investigation of inherited red cell membrane disorders in animals.

HEMOLYTIC ANEMIAS CAUSED BY HEREDITARY RED CELL MEMBRANE DEFECTS

The membrane protein-protein and protein-lipid interactions are the critical determinants of red cell morphology and mechanical stability, as evidenced by the numerous hereditary RBC disorders in humans attributed to mutations of the membrane.^{2,51-53,73,74} These interactions are divided into two categories (Fig. 29.1): (1) vertical interactions involving the band 3-ankyrin-spectrin and glycophorin C-protein 4.1-spectrin binding, which attach the spectrin-actin network to the plasma membrane and stabilize the lipid bilayer, and (2) horizontal interactions involving spectrin dimer-dimer association (tetramer formation), and contact of the distal ends of spectrin with F-actin by the aid of protein 4.1 and adducin within the junctional complex. In general, the loss of vertical linkage between membrane skeleton and lipid bilayer causes decreased membrane cohesion leading to membrane loss (e.g. hereditary spherocytosis). Weakening of horizontal linkages

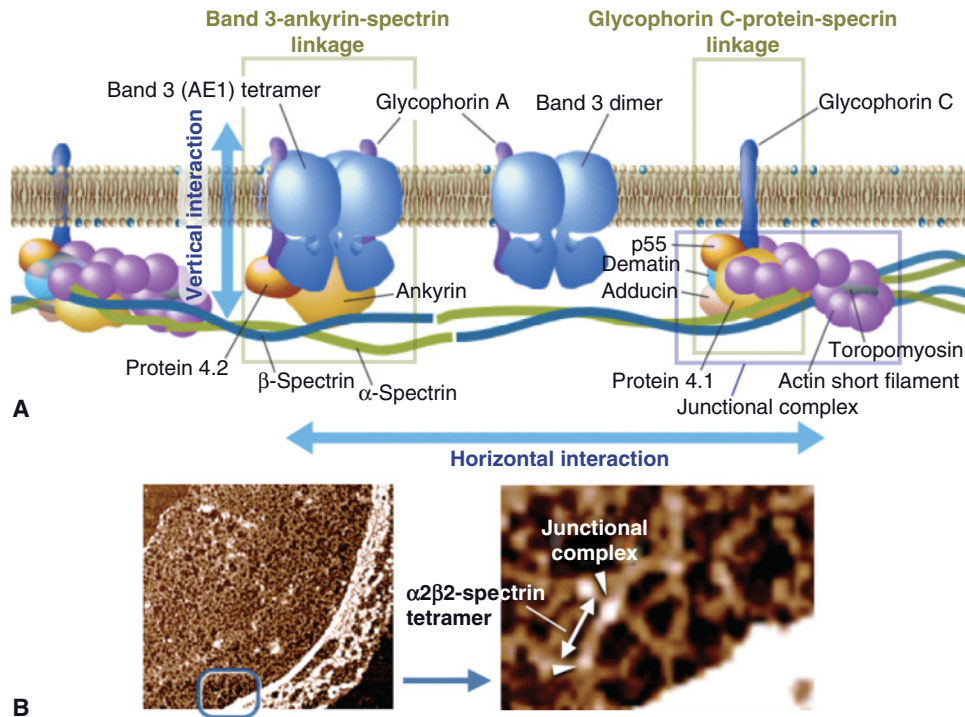


FIGURE 29.1 A schematic diagram illustrating structural and functional organization of RBC membrane proteins. Membrane protein-protein associations are divided into two categories: (1) vertical interactions involving band 3-ankyrin-spectrin linkage and glycophorin C-protein 4.1 (P4.1)-spectrin linkage, and (2) horizontal interactions involving the spectrin heterodimer contact (tetramer formation) and association of spectrin tetramer at the distal ends with F-actin at the junctional complex consisting of actin, P4.1, and adducin. In general, a defect of vertical interactions leads to hereditary spherocytosis, whereas a defect of horizontal interactions causes hereditary elliptocytosis and pyropoikilocytosis.

between skeletal proteins results in decreased membrane mechanical stability leading to membrane loss (e.g. hereditary elliptocytosis).^{1,51} The severity of the anemia is directly related to the extent of the membrane surface area loss in both of these defects; loss of membrane not only compromises the ability of the RBC to deliver oxygen to the tissues, but also results in premature removal from the circulation by the spleen. In human patients, splenectomy results in correction of the anemia.¹ The reader is directed to Chapter 20 for details of the structural organization of the normal RBC membrane.

HEREDITARY SPHEROCYTOSIS

The cardinal features of hereditary spherocytosis (HS) are hemolytic anemia of varying severity, spherocytosis, increased RBC osmotic fragility, and splenomegaly.⁵⁹ Pathophysiology of HS involves two major factors: intrinsic membrane defect, and selective sequestration of HS cells in the normal spleen. Hence, anemia can be corrected by splenectomy in human HS patients. Typical forms of HS need to be distinguished from other hemolytic anemias manifesting moderate to small numbers of spherocytes, such as autoimmune hemolytic anemia by Coombs' test as well as unstable hemoglobin and oxidative damage by Heinz body screening. HS is now

considered to be a disorder of vertical interactions of the membrane proteins, although the primary molecular defects are heterogeneous including deficiencies or dysfunctions of spectrin, ankyrin, band 3, and protein 4.2. Consequently, the lipid bilayer is destabilized, leading to membrane and surface area loss, and spherocyte formation.^{25,53,74}

Hereditary Band 3 Deficiency in Cattle (Band 3^{Bov.Yamagata})

Band 3 (anion exchanger 1, AE1) is the most abundant transmembrane protein in mammalian RBCs.⁵⁶ Hereditary band 3 deficiency in Japanese black cattle (band 3^{Bov.Yamagata}) is associated with HS and is inherited by an autosomal dominant trait. Homozygous affected animals totally lack band 3 due to a nonsense mutation Arg664 → Stop (R664X) of the *AE1* gene (*SLC4A1*)²⁹ and show mild to moderate, chronic hemolytic anemia (hematocrit, 25–35%); slight acidosis; and growth retardation. Carrier cattle heterozygous for the R664X mutation also have abnormal RBC morphology and impaired anion transport activity due to partial deficiency of RBC band 3 (about 30%). However, spherocytosis in heterozygotes is mild, and the hemolysis is well compensated. In heterozygous animals, mutant band 3 is rapidly degraded in the endoplasmic reticulum without

translocation to the plasma membrane and there is selective reduction of mutant band 3 mRNA.^{30,31}

Genotypes for R664X mutation are easily determined using genomic DNA as the template by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)^{29,30} or PCR-single strand conformation polymorphism (PCR-SSCP) techniques. A protocol to determine the genotype for band 3^{Bov.Yamagata} and the sex of embryos before embryo transfer has been developed.³⁷ The ancestral origin of this genetic defect has not yet been identified.

Band 3 has two major functions:^{34,56,71,72} (1) it mediates a rapid $\text{Cl}^-/\text{HCO}_3^-$ exchange across the plasma membrane to increase five-fold the capacity of the blood to carry CO_2 from tissues to lungs and maintains blood acid-base homeostasis together with the renal band 3 function; and (2) it may also participate in maintaining mechanical properties of RBC membranes by forming the band 3-ankyrin-spectrin complex. Various mutations leading to disorders and partial deficiency of RBC 3 associated with abnormal RBC morphology (spherocytosis and ovalocytosis) have been reported in humans,^{32,35,53} but none of them exhibited complete lack of the protein or its function.

The most surprising finding, therefore, is that cattle with total lack of band 3 survived to and thrived in adulthood. They suffer from extremely severe hemolytic anemia shortly after birth and exhibit jaundice and splenomegaly. The mortality rate is high during this period, particularly in the first week after birth. Once they overcome this neonatal crisis, jaundice subsides and hemolysis becomes modest. Until 1998, a half dozen homozygous affected cattle older than 3 years of age had been found. One of three affected females had two normal parturitions. The studies on bovine hereditary band 3 deficiency have demonstrated the importance of band 3 in RBC morphology and homeostasis.

Red blood cells from homozygotes of band 3^{Bov.Yamagata} are also deficient in spectrin, ankyrin, actin (by 20–50%), and protein 4.2, resulting in a distorted and disrupted membrane skeletal network (Fig. 29.2). Their RBC membranes are extremely unstable and demonstrate the spontaneous loss of surface area by invagination, vesiculation, extrusion of microvesicles, and fragmentation, thereby leading to the formation of spherocytes with irregular contours, gouging, and pitting (Fig. 29.3). RBCs from homozygous and heterozygous cattle constantly show considerably increased osmotic fragility with 50% hemolysis at 0.75% and 0.65–0.70% NaCl, respectively (normal, 0.45–0.55% NaCl), demonstrating that the surface area/volume ratios of RBCs from both homozygotes and heterozygotes are remarkably reduced due to the mechanical instability of their RBC membranes (Fig. 29.4). Despite these severe changes, the affected animals show no reticulocytosis and no noticeable intravascular hemolysis. However, the protein 4.1a/4.1b ratio, which is a good marker of RBC aging,^{24,27,28} is remarkably reduced and paralleled by increased erythropoiesis. These findings demonstrate the functional importance of band 3-ankyrin-spectrin

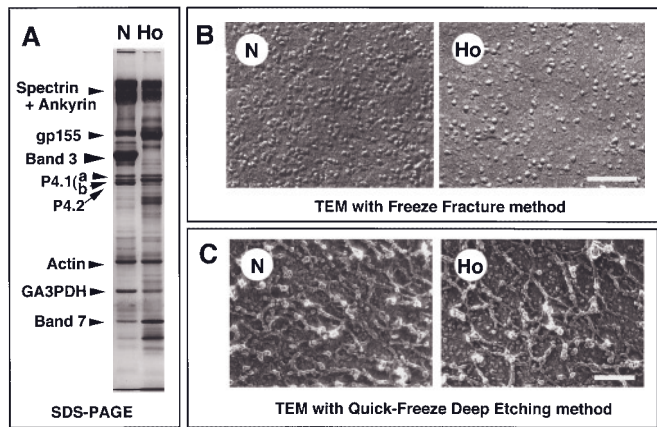


FIGURE 29.2 Abnormal RBC membrane structures in bovine band 3 deficiency (band 3^{Bov.Yamagata}). (A) SDS-PAGE profiles of RBC membrane proteins from the normal (N) and the homozygote for R664X mutation (Ho). Note that spectrin (+ankyrin) and actin levels are reduced compared to protein 4.1. Protein 4.2 is almost missing. A 66-kDa protein only found in the homozygote was albumin. gp155 indicates a transmembrane protein characteristic to ruminant RBCs.⁶⁵ (B) Markedly reduced number of intramembrane particles in homozygous RBCs (Ho) compared to normal RBC on electron micrographs by the freeze fracture method. (C) Disrupted membrane skeletal network in band-3-deficient RBCs visualized by the quick-freeze deep etching method. The membrane skeletons in band-3-deficient RBCs (Ho) are disrupted and distorted with filaments of uneven length and width compared with the well-organized normal RBCs (N). Scale bars = 0.1 μm.

association in maintaining mechanical stability of the membrane⁴¹ but not in assembly of the membrane skeletal architecture; they also indicate that accelerated and continual destruction of RBCs occurs in homozygous cattle and also in heterozygotes though it is less severe.

Total deficiency of band 3 also results in defective $\text{Cl}^-/\text{HCO}_3^-$ exchange. The Cl^- influx into the RBCs from homozygotes requires approximately 2 hours to reach transmembranous equilibrium even at 37°C. Band 3 deficiency causes mild acidosis with decreases in the HCO_3^- concentration and total CO_2 in the blood. However, these values remain in the normal range. As CO_2 in blood rarely reaches saturation, it is suggested that the additional CO_2 carrying capacity facilitated by band 3 is probably not as critical as has been believed previously,^{33,70} except under high stress conditions such as vigorous exercise or high altitude.

Transgenic mice with complete band 3 deficiency have similar RBC features.^{57,68} These studies demonstrate that band 3 indeed contributes to RBC membrane organization, CO_2 transport, and acid-base homeostasis. In band 3 knockout mice, glycolytic enzymes that normally exist as multienzyme complexes on the inner surface of RBC membranes, are not membrane-associated but distributed throughout the cytoplasm. The assembly of glycolytic enzymes on the membrane is likely a general phenomenon of mammalian RBCs and stability of these interactions depends primarily on band 3.⁶

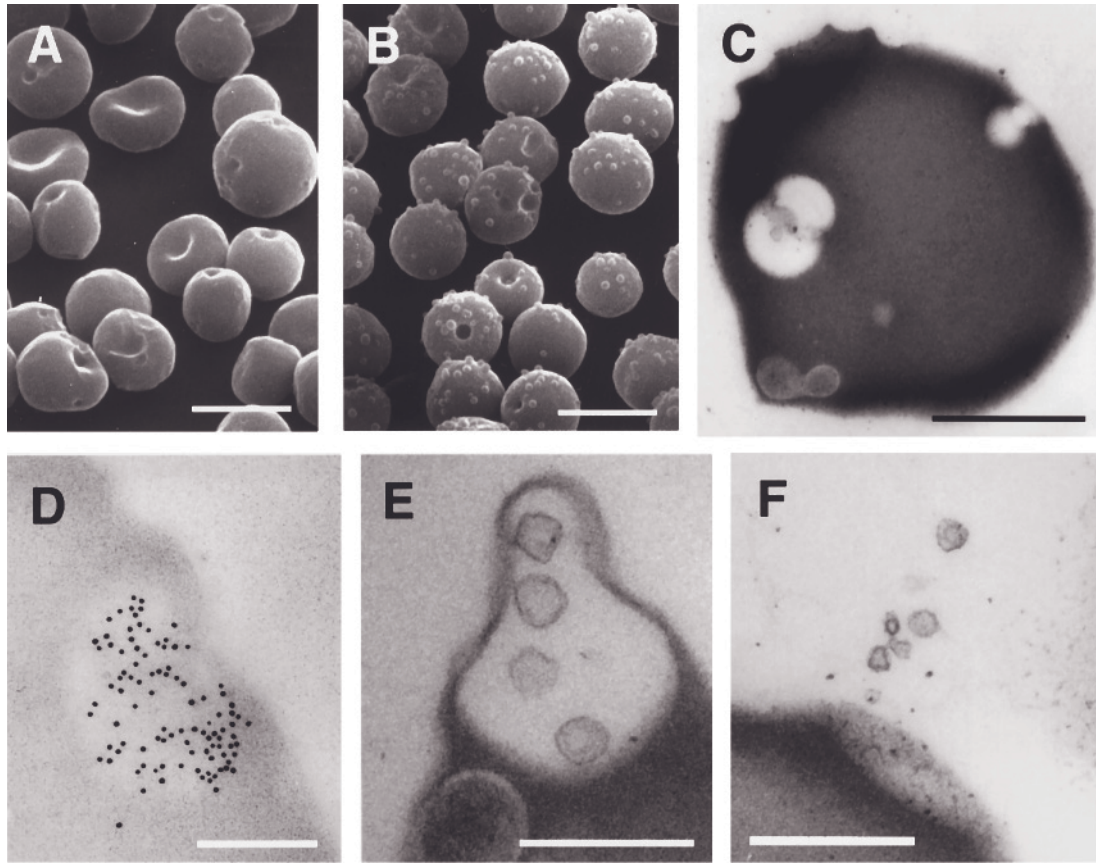


FIGURE 29.3 Morphologic anomaly in bovine band 3 deficiency. Scanning (A, B) and transmission (C–F) electron micrographs of RBCs from a homozygous animal of band 3^{Bov.Yamagata}. (A) The proband RBCs look like potatoes and greatly vary in size, principally being spherocytic and stomatocytic with irregular contours. (B) When blood is allowed to stand for several hours at ambient temperature, numerous small globules are observed on the surface. (C–F) Marked endocytosis-like invagination, exocytosis-like projections, fusion of vesicles inside the cell, and extrusion of microvesicles. The vesicles in the cytoplasm contain plasma proteins as determined by immunoelectron microscopy using anti-bovine albumin antibodies (D). Scale bars = 5 μm (A and B), 1 μm (C), and 0.2 μm (D–F).

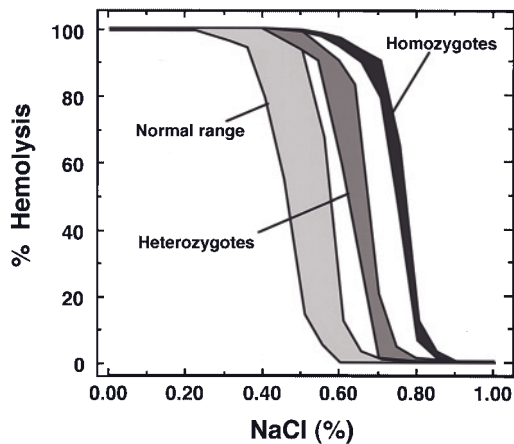


FIGURE 29.4 Increased RBC osmotic fragility in bovine band 3 deficiency. Data for normal cattle ($n = 15$) as well as homozygotes ($n = 4$) and heterozygotes ($n = 8$) of band 3 deficiency.

Spectrin and Ankyrin Deficiencies in Animals

Several mutations that affect components of the RBC membrane skeleton have also been reported in mice.^{3,79} These include spectrin deficiencies in house^{21,42} and deer mice⁶³ as well as ankyrin deficiency in nb mice⁷⁹ with moderate to life-threatening hemolytic anemia and fragile short-lived spherocytes.

A quantitative spectrin deficiency with an autosomal dominant inheritance was reported in Dutch Golden Retrievers.⁶⁶ The affected dogs had markedly reduced RBC spectrin concentration that was 50–65% of unaffected dogs and exhibited increased RBC osmotic fragility. However, the disorder was not associated with either spherocytosis or elliptocytosis. The exact mechanism for this occult spectrin deficiency without morphological anomaly remains to be clarified.

Bovine α - and β -spectrin genes possess polymorphisms leading to generation of spectrin proteins with different amino acid sequences. One of these genotypes appears to cause mild reduction in RBC spectrin contents without morphological lesions and

probably has a modulatory role in severity of RBC phenotypes in band 3^{Bov.Yamagata} (M. Inaba, unpublished observation).

HEREDITARY ELLIPTOCYTOSIS

The principal lesion of human hereditary elliptocytosis (HE) involves heterogeneous defects in horizontal membrane protein interactions (Fig. 29.1) resulting in a mechanically unstable membrane (e.g. abnormal spectrin structure affecting the spectrin heterodimer contacts, deficiency or dysfunction of protein 4.1).^{51,53,74} Common HE is morphologically characterized by elliptocytes and rod-shaped RBCs in some patients. In severe cases, aberrant disruption of the horizontal interactions results in fragmentation of RBCs, leading to hereditary pyropoikilocytosis (HPP). Ovalocytosis, spherocytic HE, is a rare condition in which both round oval RBCs lacking central concavity and spherocytes are present on the blood film. It is likely that elliptocytes and poikilocytes are permanently stabilized in their abnormal shape. The weakened horizontal connections facilitate reorganization of skeleton, which follows axial deformation of cells by a prolonged shear stress.^{51,53,74} Although camelid RBCs are elliptocytic by nature, the molecular basis and physiologic importance of this unique feature remains unknown.

Hereditary Protein 4.1 Deficiency in Dogs (HE in Dogs)

Canine HE was discovered by Smith et al.⁶⁷ The proband exhibited elliptocytosis, membrane fragmentation, microcytosis, and poikilocytosis without anemia (Fig. 29.5). RBC mechanical stability was markedly decreased, and osmotic fragility was remarkably increased. The

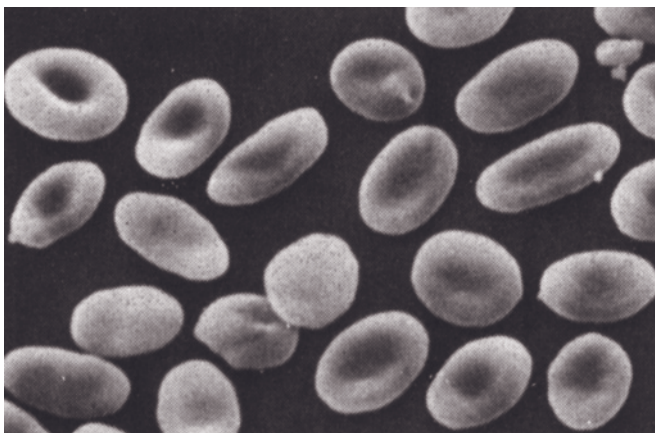


FIGURE 29.5 Scanning electron micrograph of RBCs from the proband with hereditary elliptocytosis. The proband RBCs reveal biconcave elliptocytes. (Reproduced from Smith JE, Moore K, Arens M, Rinderknecht GA, Ledet A. Hereditary elliptocytosis with protein band 4.1 deficiency in the dog. *Blood* 1983;61:373-377, with permission.) Scale bar = 5µm.

proband RBC membranes were deficient in protein 4.1 (4.1a and 4.1b). The parents of the proband had decreased amounts (approximately 50% of normal) of protein 4.1 and some elliptocytes.

Protein 4.1 forms a tertiary complex with spectrin and actin within the junctional complex. This spectrin-actin binding region was mapped to the 10-kDa domain⁹ containing amino acid sequences encoded by a 21-amino acid alternative exon and a 59-amino acid constitutive exon.⁷ Immunoblotting of RBC membrane proteins from the proband showed the presence of a small amount of 76-kDa polypeptide lacking the 21 amino acid segment and a very faint band of normal protein 4.1. RT-PCR analysis and sequencing of cloned reticulocyte protein 4.1 cDNA consistently showed that mRNA with a deletion of the alternative exon encoding 21 amino acid peptide was the predominant form with only a small quantity of normal mRNA. Therefore, the functional defect in the HE dog is likely due to the combined influences of two factors: a quantitative deficiency of protein 4.1 and a failure to activate efficiently expression of an alternatively spliced exon encoding 21 amino acids in the spectrin-actin binding region during erythropoiesis.⁸ The primary cause leading to inefficient RBC-specific alternative splicing has not been defined.

Spectrin Anomaly with Elliptocytosis in a Dog

An elliptocytosis was recently reported in a mixed-breed dog.¹¹ Although the elliptocytosis was asymptomatic and was not associated with hemolytic anemia, the dog's RBCs had decreased deformability and stability when subjected to shear stress. This is consistent with non-hemolytic HE in humans due to structural abnormality in either the α - or β -spectrin. Molecular analysis revealed that the elliptocytosis was probably due to a β -spectrin mutation in codon 2110 (T2110M), a region of spectrin that is critical for the self-association of spectrin dimmers. This would be expected to result in reduced formation or reduced stability of tetramers.

HEREDITARY STOMATOCYTOSIS

Stomatocytes are uniconcave or bowl-shaped RBCs in suspension but have a slit-like appearance, an artifact on dried blood films (Fig. 29.6). Two distinct phenotypes having abnormalities in membrane cation permeability leading to changes in RBC volume have been identified in humans: dehydrated hereditary stomatocytosis (DHS) and overhydrated hereditary stomatocytosis (OHS).^{1,51} DHS is associated with a well-compensated hemolytic anemia with less than 10% stomatocytes usually seen on blood smears. In contrast, stomatocytes are a major feature of RBC morphology in OHS. An important characteristic of these stomatocytosis patients is a marked predisposition to thrombocytosis and development of hypercoagulability after splenectomy.¹

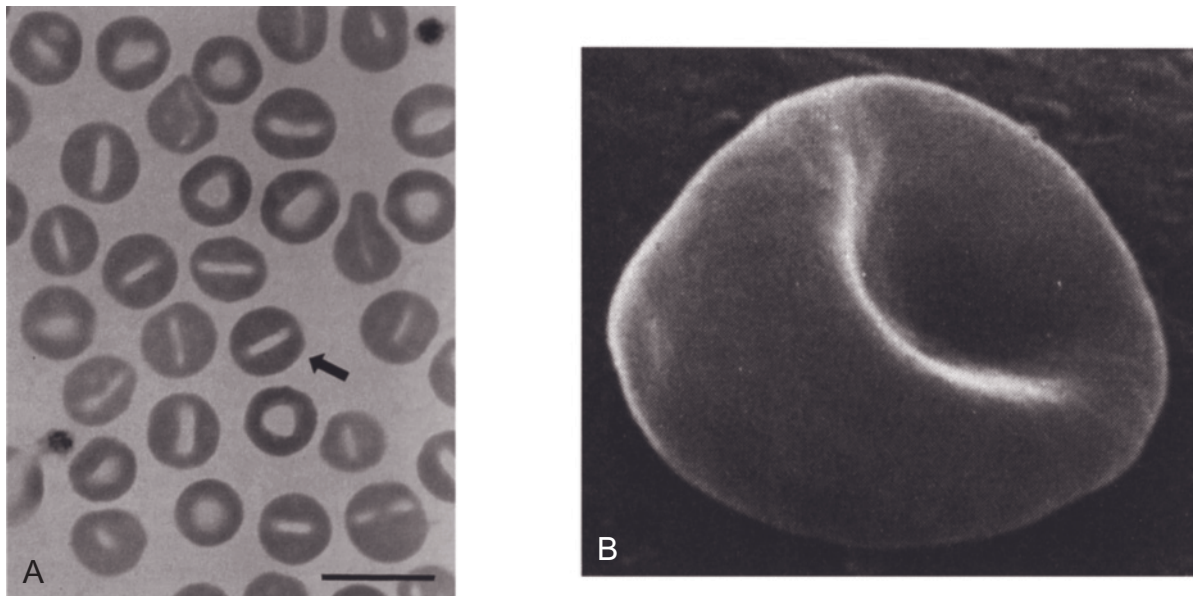


FIGURE 29.6 Erythrocyte morphology of canine hereditary stomatocytosis (hydrocytosis). (A) Numerous stomatocytes (arrow) with a slit-like appearance are present on a Wright-Giemsa-stained blood smear from a Miniature Schnauzer. (Reproduced from Brown DE, Weiser MG, Thrall MA, Giger U, Just CA. Erythrocyte indices and volume distribution in a dog with stomatocytosis. *Vet Pathol* 1994;31:247–250, with permission.) (B) Scanning electron micrograph of a typical stomatocyte from an affected Alaskan Malamute. (Reproduced from Pinkerton PH, Fletch SM, Brueckner PJ, Miller DR. Hereditary stomatocytosis with hemolytic anemia in the dog. *Blood* 1974;44:557–567, with permission.) Scale bar = 10 μ m. (Courtesy of Mitsumi Inaba and Joanne B. Messick.)

OHS (hydrocytosis) is a heterogeneous group of disorders in humans characterized by moderate to severe hemolytic anemia with stomatocytes, an elevated mean corpuscular volume, and a reduced mean corpuscular hemoglobin concentration. The principal lesion involves a remarkable increase of Na^+ influx into RBCs, resulting in a marked increase of intracellular Na^+ and water content and a corresponding decrease of K^+ .^{50,86} The molecular basis of this permeability defect is unknown. Human OHS is frequently associated with reduced contents of a 31-kDa integral membrane protein 7.2b (stomatins), which may function in regulation of cation transport and a stretch- or pressure-sensitive system, in the RBC membrane from OHS patients.^{16,40,69} It is reported that stomatin-actin association is necessary for maintaining the structure and modulating the function of stomatin in RBCs.⁸⁰

Hereditary Stomatocytosis in Dogs and Cats

Hereditary stomatocytosis (HSt) is recognized in dogs and cats with undefined etiologies. All disorders appear to be transmitted as autosomal recessive traits.

HSt in Miniature and Standard Schnauzers

Stomatocytosis inherited by autosomal recessive trait has been reported in Miniature and Standard Schnauzers (Fig. 29.6A) without clinical signs of disease.^{4,5,20} Hereditary stomatocytosis in Schnauzers is characterized by macrocytosis, relatively high packed cell volume, remarkably decreased mean corpuscular

hemoglobin concentration, and increased osmotic fragility. Erythrocyte survival is only slightly shortened. Affected Miniature Schnauzers are of normal stature. Stomatins are not deficient from the erythrocyte membrane in affected Standard Schnauzers.⁵⁴

HSt in Chondrodysplastic Alaskan Malamute Dwarf Dogs

In Alaskan Malamutes, chondrodysplasia (short-limbed dwarfism) occurs along with stomatocytosis^{14,15} (Fig. 29.6B). The affected Malamutes have macrocytosis, decreased mean corpuscular hemoglobin concentration, increased osmotic fragility, shortened RBC survival, reticulocytosis, erythroid hyperplasia, and increased iron turnover.⁵⁸ Although heterozygous carriers have minor changes in their RBCs, they have a normal RBC lifespan and no dwarfism. RBC Na^+ concentration and water content are increased in affected dogs.

The pathogenesis of stomatocyte formation in Malamutes and Schnauzers is attributed to an increase in monovalent cation and, consequently, increased water content of RBCs as is reported in human HSt. However, the exact nature of membrane defects leading to the changes in RBC indices in these breeds is obscure.

Familial Stomatocytosis-Hypertrophic Gastritis in Dogs

Familial stomatocytosis-hypertrophic gastritis is a multiorgan disease with hemolytic anemia, HSt, and hyper-

trophic gastritis described in the Drentse Partrijshond breed.^{64,65} The main clinical signs are diarrhea, jaundice, and ataxia and paresis of the pelvic limbs. Pathologic findings involve hypertrophic gastritis, progressive liver disease, renal cysts in aged subjects, and polyneuropathy.⁶⁴

Erythrocytes from affected dogs show increased osmotic fragility. In contrast to Malamutes and Schnauzers with stomatocytosis, RBCs from affected Partrijshonds have normal mean corpuscular volume, slightly increased cell water with basically normal content, and fluxes of Na and K, suggesting a different mechanism for stomatocyte formation.⁶⁵ Affected dogs have normal RBC membrane protein profile, but a decrease of phosphatidylcholine with altered fatty acid composition and simultaneous increase of sphingomyelin in both RBCs and plasma. It is suggested that this polysystemic disease is a disorder of lipid metabolism in which defective membrane function and RBC shape change are induced by abnormal phospholipid composition of the plasma. This is supported by a shortened half-life of RBCs from normal dogs after transfusion into dogs with this syndrome. Stomatocytic shape change may be attributed to membrane surface loss, presumably due to abnormal phospholipid composition of the bilayer. The exact relation between anomaly in phospholipid and hypertrophic gastritis and the primary cause of this syndrome are unknown.

Hemolytic Anemia with Increased Erythrocyte Osmotic Fragility in Cats

A hereditary RBC defect is suspected in Abyssinian and Somali cats with Coombs' negative hemolytic anemia.^{38,39} The affected cats exhibited recurrent anemia (hematocrit, 15–25%); severe splenomegaly with extra medullary hemopoiesis, hemosiderosis, congestion, and lymphoid hyperplasia; loss of body mass; macrocytosis; and a few stomatocytes. The anemia was variably regenerative. The osmotic fragility of their RBCs was markedly increased. Splenectomy partially corrected the anemia and prevented hemolytic crises, but long-term survival remains unknown.

DIAGNOSTIC APPROACHES TO ERYTHROCYTE MEMBRANE DEFECTS

Genetic aberrations of RBC membranes described above are feasibly surveyed by evaluating RBC morphology, osmotic fragility, and RBC parameters. The osmotic fragility test is a simplified means to estimate the surface area/volume ratio of RBCs. It is most valuable in the diagnosis of HS but is also useful in evaluation of most forms of HE and overhydrated HSt (hydrocytosis). Several laboratory tests, such as Coombs' test and Heinz body screening, may be required to eliminate a possibility that hemolytic anemia and abnormal RBC shapes result from extrinsic factors. As exemplified for bovine HS in Figure 29.2A, SDS-PAGE analysis followed by some immunochemical, biochemical, and biophysical

techniques often provides insights into primary defects of membrane skeletal and integral proteins resulting in HS, HE, and HPP.

MEMBRANE TRANSPORT DEFECTS

Deficiency and dysfunction of membrane transport systems may affect RBC homeostasis. Particularly, defects in transport of amino acids involved in glutathione metabolism have been reported to generate hemolytic anemia when RBCs are exposed to extrinsic factors including oxidants.

Amino Acid Transport Deficiency in Animals

Red blood cell glutathione deficiency, inherited as an autosomal recessive trait, occurs in Finnish Landrace sheep.⁷⁵ Affected animals are not anemic but have shortened RBC lifespan.⁷⁶ This is possibly caused by increased oxidant sensitivity as exemplified by Heinz bodies. Affected sheep are more likely to become anemic after the administration of oxidants *in vivo*.⁷⁸ RBCs from the affected animals are defective in the transport system for various amino acids including cysteine.^{84,85} Consequently, cysteine uptake and glutathione synthesis are limited, and glutathione concentrations in RBCs are decreased to approximately 30% of normal. The transport deficiency appears to develop during reticulocyte maturation.⁷⁷

A similar defect of amino acid transport is found in about 30% of thoroughbred horses¹³ but seems to cause no clinical signs. The lesion appears to result in increased amino acid levels and glutathione deficiency in some cases.

High Membrane Na,K-ATPase Activity in Dogs

Although canine reticulocytes have a considerable amount of membrane Na,K-ATPase (Na/K-pump) activity, its activity is rapidly lost during maturation into mature RBCs.⁴⁶ Proteolytic degradation²³ and extrusion of vesicles (exosomes)³⁶ are likely involved in this process. As a consequence, dogs usually have RBCs with low K⁺ and high Na⁺ concentrations (LK RBCs).⁵⁵ However, some Japanese Shiba and mongrel dogs have HK RBCs with high K⁺ and low Na⁺ concentrations, because the Na,K-ATPase protein and its activity are retained in mature RBCs.^{23,41,45} This HK phenotype representing immaturity of erythroid precursor cells^{26,47} is inherited in an autosomal recessive manner and has also been found in Japanese Akita¹⁰ and several breeds of Korean dogs.^{18,49} Although dogs with HK RBCs are not anemic, their RBCs have shortened lifespans,⁴⁷ increased osmotic fragility, increased mean corpuscular volume, and normal mean corpuscular hemoglobin values, suggesting an increase in RBC water.⁴⁵ The molecular basis for RBC HK and LK phenotypes is unknown. Due to the leak of K⁺ from RBCs, blood from HK dogs may cause pseudohyperkalemia *in vitro* after storage or on delaying plasma or serum separation.¹⁰

Thus, care should be taken, particularly when stored blood from HK dogs is used for transfusion.

Canine RBCs possess a high-affinity Na⁺-dependent transport system for glutamate and aspartate^{12,22} which resembles the kinetic and pharmacologic properties of the transporter in the brain.⁶⁰⁻⁶² The increased concentration gradients of Na⁺ and K⁺ across the membrane produced by the presence of Na,K-ATPase with high activity accelerates transport of glutamate and aspartate into HK RBCs.²³ The concentration of reduced glutathione is increased 5–7 times that of normal because the feedback inhibition of γ -glutamylcysteine synthetase by glutathione is released by glutamate accumulated in these cells at about 90 times that in normal RBCs.⁴⁴ Some variant dogs of HK phenotype that lack the increase of RBC glutathione have been reported,^{17,19} suggesting that several independent mutations have emerged in these breeds.⁶¹

The accumulation of glutathione in the canine HK RBCs only provides improved protection against oxidative damage induced by acetylphenylhydrazine, but these RBCs are more susceptible to oxidative damage induced by 4-aminophenyl disulfide,⁴⁸ onions,^{43,81} and sodium n-propylthiosulfate,⁸³ one of the hemolytic thiosulfate compounds isolated from onions.⁸² The increased glutathione concentration accelerates the generation of superoxide through its redox reaction with the aromatic disulfide,⁴⁸ but the exact mechanism by which HK RBCs are more sensitive to the thiosulfates remains to be clarified.

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Congenital Dyserythropoiesis

DOUGLAS J. WEISS

Poodle Macrocytosis
 Inherited Selective Malabsorption of Cobalamin
 Clinical Condition
 Congenital Dyserythropoiesis, Polymyopathy, and
 Cardiac Disease in English Springer Spaniels

Hereditary Stomatocytosis in Alaskan Malamutes (see
 Chapter 29)
 Congenital Anemia, Dyskeratosis, and Progressive
 Alopecia in Polled Hereford Calves

Acronyms and Abbreviations

RBC, red blood cell; RDW, RBC distribution width.

Congenital conditions associated with dysplastic features in red blood cells (RBCs) and bone marrow include Poodle macrocytosis,^{2,8} inherited selective malabsorption of cobalamin,^{4,5,7} congenital dyserythropoiesis, polymyopathy, and cardiac disease in English Springer Spaniels,⁶ hereditary stomatocytosis in Alaskan Malamutes (see Chapter 29), and congenital anemia, dyskeratosis, and progressive alopecia in polled Hereford calves.^{9,10} In general, these conditions are characterized by dysplastic changes in RBCs or erythroid precursor cells associated with ineffective erythropoiesis.¹¹ The mechanisms associated with accelerated intra-marrow cell death are discussed in Chapter 64.

POODLE MACROCYTOSIS

Poodle macrocytosis is a familial condition that occurs mostly in Toy and Miniature Poodles.^{2,8} The abnormality affects both sexes. Affected dogs do not have associated clinical signs, are not anemic, and do not have reticulocytosis. The most distinctive feature in the blood is marked macrocytosis with mean cell volumes varying between 85 and 105 fL.^{2,8} Metarubricytosis is a frequent finding and some of these cells show nuclear-cytoplasmic asynchrony. Additionally, Howell-Jolly bodies are increased in number and tend to be large and multiple (Fig. 30.1). Bone marrow is characterized by marked dyserythropoiesis and the morphologic changes resemble those associated with vitamin B₁₂ deficiency. Features

of dyserythropoiesis include megaloblasts, binucleation, multinucleation, irregular nuclear shapes, nuclear fragmentation, nuclear-cytoplasmic asynchrony, and nuclear bridging (Fig. 30.2).² Nuclear bridging is a unique feature seen in Poodle macrocytosis that is rarely seen in other congenital or acquired types of dyserythropoiesis or in myelodysplastic syndromes (Fig 30.3). Mitotic figures are increased in number.

The nature of the hematopoietic defect has not been investigated. Bone marrow cytologic abnormalities in Poodle macrocytosis have some similarity to those seen in congenital dyserythropoietic anemia type I of children as well as those in Hereford cattle with congenital anemia, dyskeratosis, and progressive alopecia.^{5,9,10}

INHERITED SELECTIVE MALABSORPTION OF COBALAMIN

An inherited selective malabsorption of cobalamin (vitamin B₁₂) has been identified in Giant Schnauzers and has been reported in Border Collies, and in a Beagle and a cat.^{3,4,7} Cobalamin is obtained in all species by absorption from the intestinal tract. Cobalamin can be produced by microorganisms in the gastrointestinal tract of ruminants but must be liberated from digestion of foodstuffs by other species. Cobalamin is essential as a cofactor for the activity of the enzymes methylmalonic-CoA mutase and methionine synthase.¹ Deficiency of methylmalonic-CoA mutase results in methylmalonic acidemia and aciduria.¹ Deficiency of methionine syn-

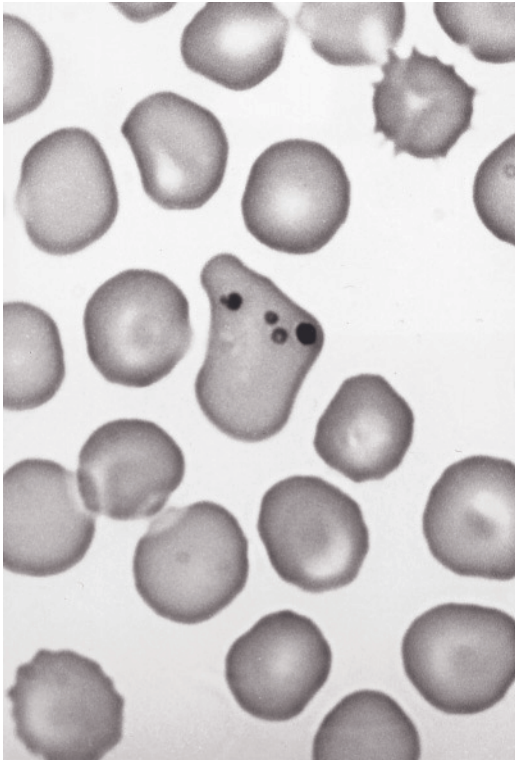


FIGURE 30.1 Blood from a dog with poodle macrocytosis. Notice the presence of an RBC with multiple large Howell-Jolly bodies.

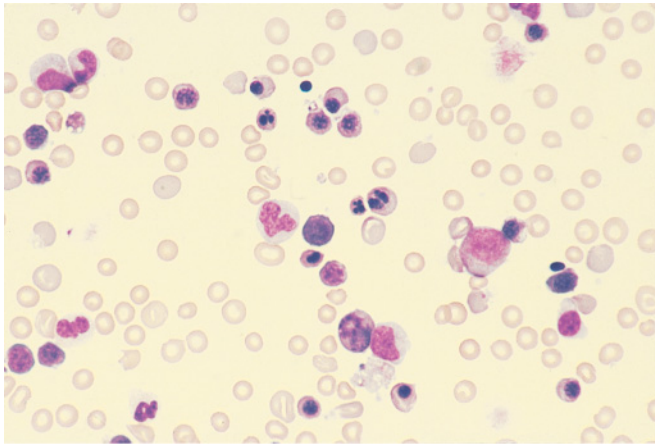


FIGURE 30.2 Erythroid precursor cells in bone marrow from a dog with Poodle macrocytosis. Notice the presence of misshapen and fragmented nuclei in rubricytes.

these causes homocysteinemia and inhibits conversion of 5-methyl-tetrahydrofolate to tetrahydrofolate.¹ Tetrahydrofolate is an essential cofactor for enzymes of purine and pyrimidine synthesis.¹ Resultant inhibition of nucleic acid synthesis is most noticeable as inhibition of hematopoiesis in adult animals.^{3,4} Inhibition of nuclear maturation typically results in a non-regenerative anemia, with megaloblastosis, neutropenia, and hypersegmented neutrophils.^{3,4} Although humans and non-human primates develop a

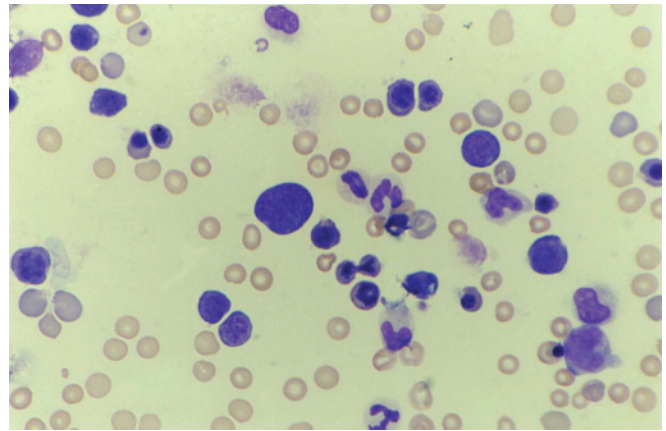


FIGURE 30.3 Bone marrow aspirate from a dog with Poodle macrocytosis. Notice the presence of a rubricyte with nuclear bridging (center of photo).

macrocytic anemia with cobalamin deficiency, the anemia in dogs, rodents, horses, pigs, and ruminants is normocytic and normochromic.^{3,4} In dogs, both large and small RBCs are present in the blood with cobalamin deficiency.^{3,4} This results in an increase in red cell distribution width (RDW) but the mean cell volume remains within the reference interval.

Clinical Condition

Inherited selective malabsorption of cobalamin in Giant Schnauzers is an autosomal recessive condition caused by lack of a receptor for intrinsic factor-cobalamin complex (i.e. cubilin) on the brush border of the small intestine and renal tubular epithelial cells.^{3,4} Affected dogs develop anorexia, lethargy, and cachexia by 2–3 months of age. Blood cell morphology is characterized initially by a mild neutropenia followed by development of a chronic non-regenerative normocytic normochromic anemia, acanthocytosis, and increased RDW.^{3,4} RBC morphology is characterized by many small RBCs, ovalocytes, and occasional megaloblasts (Fig. 30.4). Occasional hypersegmented neutrophils and giant platelets are also present. Bone marrow granulocytes are characterized by hypersegmented neutrophils and giant bands and metamyelocytes. Erythroid cells in marrow are characterized by nuclear-cytoplasmic asynchrony and abnormal nuclear chromatin clumping.

Diagnosis of cobalamin deficiency is best made by determining serum cobalamin concentrations.^{3,4} Low serum cobalamin concentration appears to be the earliest indicator of cobalamin deficiency. Increased serum methylmalonic acid and serum total homocysteine concentrations can also be measured.

Affected dogs respond well to daily injections of vitamin B₁₂.^{3,4} Alternatively, one megadose of 1 mg of vitamin B₁₂ is sufficient to maintain an affected dog in remission for 1 month. Complete resolution of hematologic signs and growth retardation occurs with parenteral administration of cyanocobalamin.

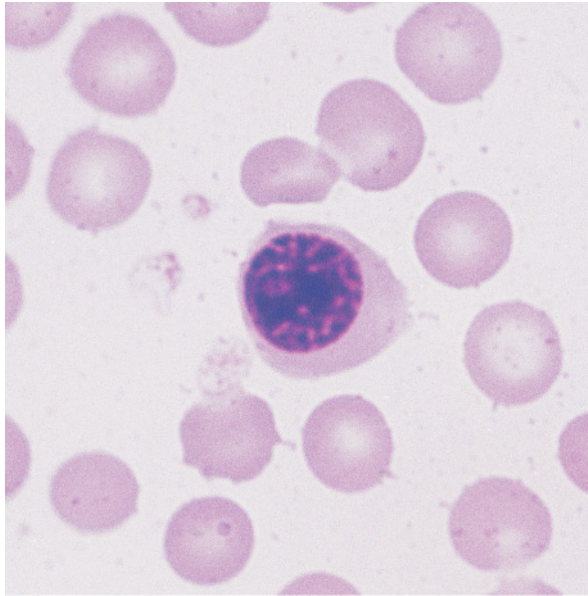


FIGURE 30.4 Blood from a dog with selective malabsorption of cobalamin. Notice the presence of a megaloblast exhibiting an immature nucleus and a fully hemoglobinized cytoplasm.

CONGENITAL DYSERYTHROPOIESIS, POLYMYOPATHY, AND CARDIAC DISEASE IN ENGLISH SPRINGER SPANIELS

Congenital dyserythropoiesis, polymyopathy, and cardiac disease has been described in three related English Springer Spaniels.⁶ Clinical signs include regurgitation, stunted growth, stiff gait, skeletal muscle wasting, cardiomegaly, megaesophagus, and generalized muscle atrophy that is most pronounced in temporal muscles. All dogs have a mild to moderate microcytic normochromic non-regenerative anemia with marked metarubricytosis. Alterations in RBC morphology included spherocytes, schistocytes, dacryocytes, codocytes, and vacuolated RBCs. Bone marrow is characterized by erythroid hyperplasia and dyserythropoiesis. Features of dyserythropoiesis include lobulated nuclei, marked binucleation, arrested mitotic figures, and cytoplasmic vacuolization. The hematologic condition remain stable but the polymyopathy is slowly progressive.

CONGENITAL ANEMIA, DYSKERATOSIS, AND PROGRESSIVE ALOPECIA IN POLLED HEREFORD CALVES

Hereford calves with congenital anemia, dyskeratosis, and progressive alopecia have a congenital syndrome characterized by dyserythropoiesis and progressive alopecia associated with dyskeratotic hyperkeratosis.^{9,10} A familial pattern has been demonstrated which is consistent with a simple autosomal recessive mode of inheritance.

Clinically, the condition is slowly progressive but is frequently fatal.⁹ Iron, cobalamin, and folate deficiencies were ruled out as causes and viral agents were not identified in affected calves. Affected calves have a macrocytic normochromic anemia with a mild reticulocytosis. Dyserythropoiesis in bone marrow is characterized by multinuclearity, nuclear bridging, irregular nuclear shapes, and irregular chromatin patterns. These dysplastic changes resemble those associated with Poodle macrocytosis and congenital dyserythropoiesis type I in humans.^{2,5}

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Anemia Caused by Rickettsia, Mycoplasma, and Protozoa

ROBIN W. ALLISON and JAMES H. MEINKOTH

Rickettsia

Ehrlichiosis

- Canine monocytic ehrlichiosis
- Canine granulocytic ehrlichiosis

Anaplasmosis

- Granulocytic anaplasmosis
 - Granulocytic anaplasmosis in dogs
 - Granulocytic anaplasmosis in horses
 - Granulocytic anaplasmosis in ruminants
- Erythrocytic anaplasmosis in ruminants

Hemotrophic Mycoplasmas (Hemoplasmas)

- Canine hemotrophic mycoplasmas
- Feline hemotrophic mycoplasmas
- Swine hemotrophic mycoplasmas
- Ruminant hemotrophic mycoplasmas
- Camelid hemotrophic mycoplasmas

Protozoa

Theileriosis

- Ruminants
- Equines
- Canines

Babesiosis

- Canines
 - Large canine *Babesia*
 - Small canine *Babesia*

Ruminants

Equines

Felines

Feline Cytauxzoonosis

Trypanosomiasis

Sarcocystosis

Haemoproteus

Plasmodium

Acronyms and Abbreviations

APBT, American Pit Bull Terrier; CME, canine monocytic ehrlichiosis; CNS, central nervous system; EDTA, ethylenediaminetetraacetic acid; FeLV, feline leukemia virus; FIV, feline immunodeficiency virus; HGE, human granulocytic ehrlichiosis; PCR, polymerase chain reaction; PCV, packed cell volume; RBC, red blood cell.

A seemingly ever-increasing number of rickettsial, mycoplasmal and protozoal agents are being recognized as the cause of anemia in veterinary species.² The anemia is frequently hemolytic in nature; however, with some organisms the anemia is non-regenerative resulting either from cytokine suppression of hematopoiesis or bone marrow pathology. Of importance to the veterinary hematologist is that many of these organisms can be visualized on blood films, providing a definitive diagnosis.

RICKETTSIA

The order *Rickettsiales* contains many organisms that cause significant hematologic disease in veterinary

species. Many of the genera in this order have undergone reclassification. The order now contains two families, *Anaplasmataceae* and *Rickettsiaceae*. The *Anaplasmataceae* are intracellular parasites that grow within a cytoplasmic vacuole in the host cell as opposed to the *Rickettsiaceae* that infect the host cell cytoplasm or nucleus and are not bounded by a vacuole.

Ehrlichiosis

Canine Monocytic Ehrlichiosis (CME)

Ehrlichia canis is the most significant cause of canine monocytic ehrlichiosis worldwide. Transmission is by tick vectors (primarily *Rhipicephalus sanguineus*) and direct blood inoculation. Organisms replicate in cells of

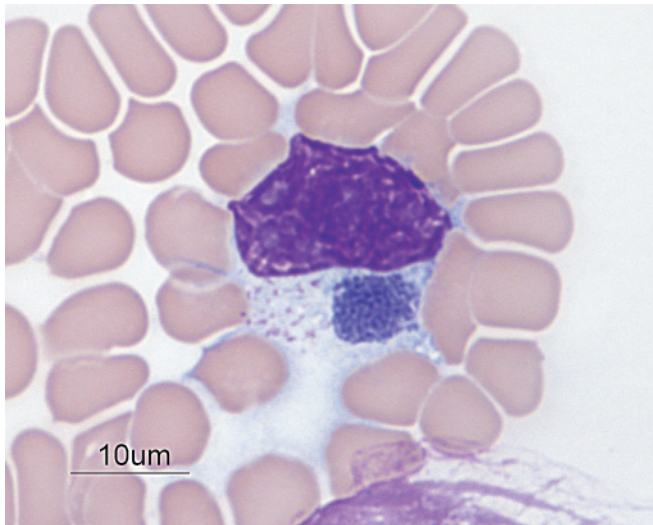


FIGURE 31.1 Blood film from a dog with *E. canis* infection. A morula is present in a disrupted large granular lymphocyte. The morula has the distinctive punctate, granular appearance. Romanowsky stain.

the mononuclear phagocyte system and can be seen in lymphocytes, monocytes or macrophages.⁴² Within mononuclear cells, organisms multiply by binary fission in a cytoplasmic vacuole. They appear by light microscopy as round, variably-sized, basophilic cytoplasmic inclusions termed morulae that have a punctate, granular appearance (Fig. 31.1). Parasitemia in peripheral blood is low and transient following initial infection. Diagnosis is generally made by serology or PCR, rather than visualization of organisms.

Hematologic findings depend on the stage of infection. Acute infection is associated with thrombocytopenia, that may be marked, and non-regenerative anemia. Leukopenia and neutropenia are less consistent. After acute infection, there is a subclinical phase during which anemia typically resolves but thrombocytopenia may be persistent. Dogs that develop the severe chronic phase typically have profound pancytopenia. Bone marrow aspirates obtained in these animals are hypocellular with marked reductions of all hematopoietic cell lines (see Chapter 19).⁸

Canine Granulocytic Ehrlichiosis

The causative agent of granulocytic ehrlichiosis is *E. ewingii*.¹ Transmitted by *Amblyomma americanum*, *E. ewingii* has been reported from the southern and southeastern United States.²¹ Polyarthrititis is a common clinical manifestation of infection.²¹ Clinical disease is considerably milder than that caused by *E. canis*. Hematologic findings include thrombocytopenia and a mild to moderate non-regenerative anemia.²¹ *E. ewingii* infection is not associated with a chronic-phase pancytopenia.

Morulae of *E. ewingii* are found predominantly in neutrophils and rarely in eosinophils. Morulae are similar in appearance to those of *E. canis*, although they sometimes have a “packeted” appearance rather than fine punctate granulation typical of *E. canis*. Organisms are seen in the blood transiently during acute infection, but parasitemia is frequently high enough to allow diagnosis by microscopy.²¹ Organisms may be identified in neutrophils of synovial fluid samples in dogs with polyarthrititis. No specific serologic test exists for *E. ewingii*, but there is cross-reaction with *E. canis* serology. Infection can also be confirmed by PCR.²¹

Anaplasmosis

Historically, members of the genus *Anaplasma* infected red blood cells (RBCs) of ruminants causing hemolytic anemia. However, with the reclassification of several *Ehrlichia* spp. as *Anaplasma* spp., members of this genus may infect RBCs (*A. marginale*), granulocytes (*A. phagocytophilum*) or platelets (*A. platys*). Anemia is not a main feature of *A. platys* infection.

Granulocytic Anaplasmosis (*A. phagocytophilum*)

Anaplasma phagocytophilum now includes the organisms previously described as *Ehrlichia phagocytophila*, *Ehrlichia equi*, and the human granulocytic ehrlichiosis (HGE) agent.

Granulocytic Anaplasmosis in Dogs *A. phagocytophilum* infection in dogs is widespread. In the United States, it occurs most commonly in the upper Midwest and along the Pacific coast.²² Clinical signs are non-specific and include fever, anorexia, depression, lymphadenopathy, and splenomegaly.²² Some dogs may manifest either a neutrophilic polyarthrititis or acute CNS signs, including seizures. Thrombocytopenia, mild to severe, is the most frequent hematologic abnormality and occurs in the majority of cases.²² Mild to moderate non-regenerative anemia may also occur.²² Morulae are similar to those of *E. ewingii* and can be seen in neutrophils at a fairly high parasitemia during the acute phase of infection.

Granulocytic Anaplasmosis in Horses *A. phagocytophilum* infection in horses has been reported from many states in the USA as well as Canada, Brazil, and Europe.³² It causes a febrile illness characterized by anorexia, limb edema, mild petechiation, and reluctance to move. The predominant hematologic finding is a profound thrombocytopenia, variable neutropenia, and mild anemia may be present.¹⁸ The parasitemia may be high with morulae readily seen in blood neutrophils during acute infection.

Granulocytic Anaplasmosis in Ruminants *Anaplasma phagocytophilum* is associated with abortion in cattle and increased susceptibility to other infections in lambs throughout Europe. Thrombocytopenia and leukopenia

are common hematologic findings, but anemia is not a prominent feature.

Erythrocytic Anaplasmosis in Ruminants

Anaplasmosis is a significant cause of disease in cattle.²⁹ Two species are described: *Anaplasma marginale* is widespread and *Anaplasma centrale* occurs in South America and South Africa. Wild ruminants are reservoirs, but do not usually manifest disease. *A. marginale* infection causes fever and mild to marked hemolytic anemia. Numerous tick species serve as vectors and mechanical transmission may also occur. The hemolysis is primarily extravascular; hemoglobinuria is not seen. Disease is most pronounced in older animals; calves 6–9 months old are relatively resistant and disease is mild. *Anaplasma centrale* is generally less pathogenic than *A. marginale*.

Organisms are visible in erythrocytes during acute infection. They are round, 0.5–1 μm, basophilic bodies frequently present on the periphery of RBCs (Fig. 31.2). They resemble Howell-Jolly bodies, from which they must be differentiated. *Anaplasma centrale* organisms are similar, but not peripherally located in RBCs. Although long thought to occur only in RBCs, *A. marginale* has been shown to invade microvascular endothelial cells both in vivo and in vitro.¹¹

After infection, parasitemia increases until the hemolytic crisis, frequently with more than 50% of RBCs infected.²⁹ After development of the anemia, parasite numbers decline due to removal of infected RBCs. Cattle that survive acute infection become chronic carriers and act as reservoirs.

Anaplasma ovis causes anaplasmosis in sheep and goats. The disease occurs in tropical and subtropical regions throughout the world. Morphologically, *A. ovis* is similar to *A. marginale*.

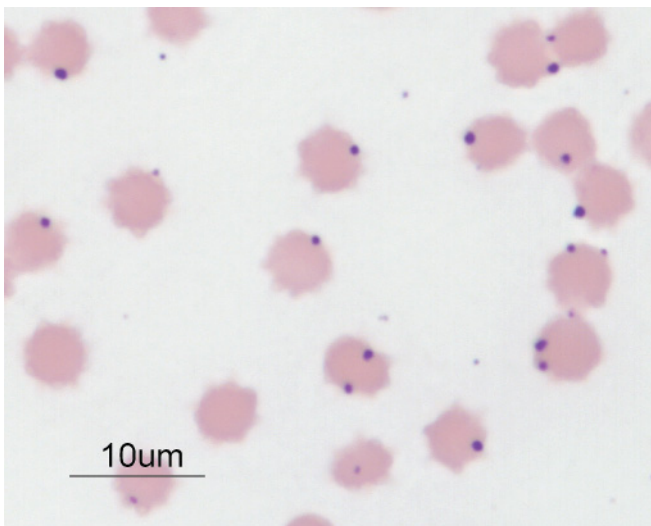


FIGURE 31.2 Blood film from a steer experimentally infected with *A. marginale*. Marked parasitemia with more than 50% of RBCs containing inclusions. Note the marginal position of most organisms. Romanowsky stain.

HEMOTROPHIC MYCOPLASMAS (HEMOPLASMAS)

Epierythrocytic parasites previously known as *Haemobartonella* and *Eperythrozoon* have been reclassified to the genus *Mycoplasma* and renamed, some with the designation *Candidatus* indicating they are incompletely described. As a group, these hemoplasmas have worldwide distribution, infect a wide variety of vertebrate animals, and share similar characteristics and morphologic features.³⁸ On routine blood films they are small basophilic round, rod, or ring-shaped organisms that appear on RBCs individually or in chains. Electron microscopy has shown them to have a single limiting membrane and lack nuclei or distinct organelles (Fig. 31.3). Organisms are typically found in depressions and invaginations in RBC membranes and appear connected to the membrane by delicate fibrils (Fig. 31.4).⁵⁷

Hemoplasmas vary in their ability to cause clinically apparent disease, but infected animals may remain carriers despite antibiotic therapy; parasitemia may re-emerge in times of stress. Hypoglycemia secondary to glucose consumption by the bacteria has been reported in heavily parasitized pigs, sheep, llamas, and calves; however, rapid bacterial glycolysis in vitro may also cause artifactually decreased blood glucose concentrations.⁹ Historically, diagnosis has relied on detection of hemoplasmas on blood films. Organisms are generally found associated with the RBC membrane, but sometimes are free in the plasma (Fig. 31.5). Hemoplasmas

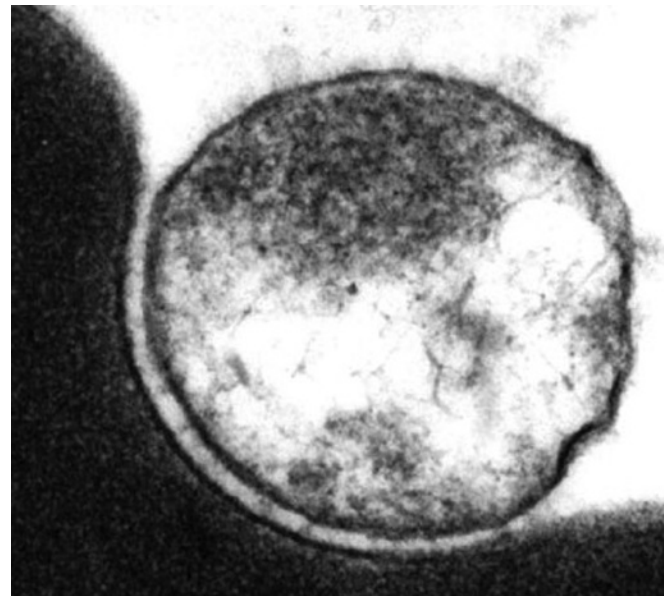


FIGURE 31.3 Transmission electron micrograph of a hemoplasma parasite (*M. haemofelis*) illustrating a single limiting membrane separating the cytoplasm of the organism from the host RBC. Delicate fibrils attach the organism to the host cell. (Reprinted from Messick JB. Hemotropic mycoplasmas (hemoplasmas): a review and new insights into pathogenic potential. *Vet Clin Pathol* 2004;33:2–13, with permission.)

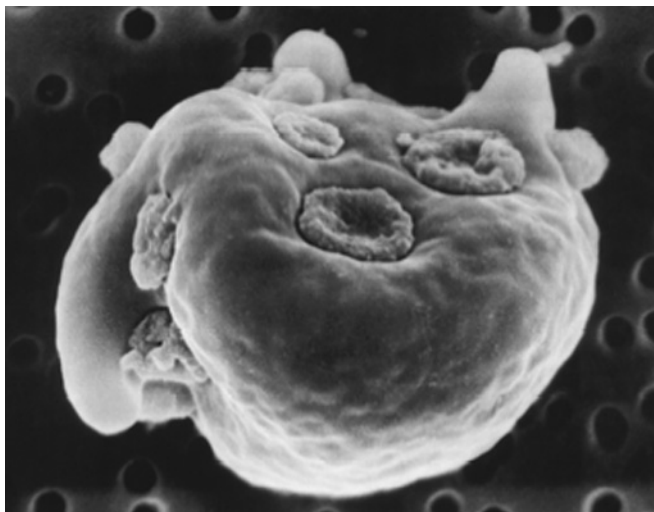


FIGURE 31.4 Scanning electron micrograph of several hemoplasma parasites (*M. suis*) within shallow depressions on the surface of an RBC. (Reprinted from Messick JB. Hemotropic mycoplasmas (hemoplasmas): a review and new insights into pathogenic potential. *Vet Clin Pathol* 2004;33:2–13, with permission.)

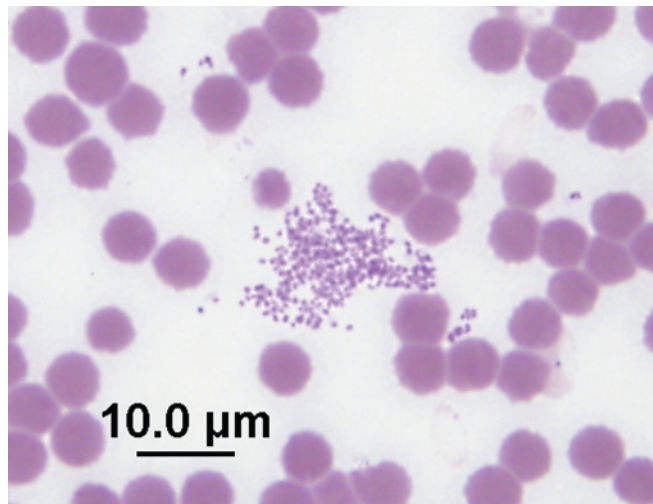


FIGURE 31.6 Blood film from a cat with *M. haemofelis* infection prepared from aged blood. Organisms have detached from RBCs and are degenerating, appearing as clumps of pink granular material in the background. Romanowsky stain.

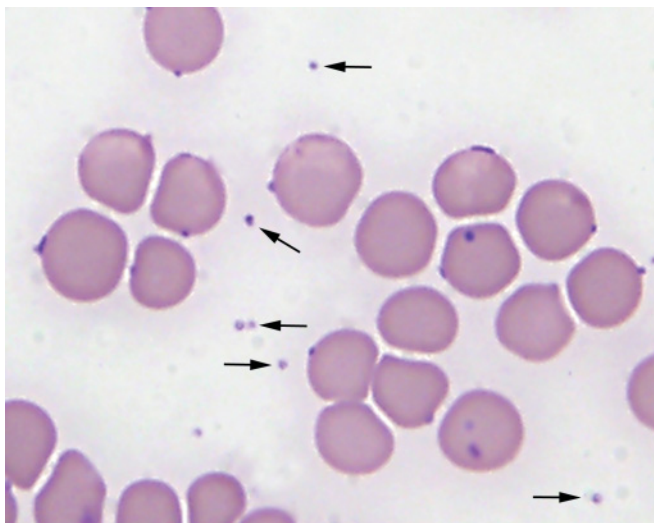


FIGURE 31.5 Blood film from a cat with *M. haemofelis* infection. Organisms are visible on the surface of RBCs and free in the background (arrows). Romanowsky stain.

dissociate from RBCs and die after a variable amount of time in EDTA.⁴³ In aged samples, dead organisms can appear as pale pink clumped granular material in the background of the blood film (Fig. 31.6). Development of sensitive PCR assays capable of discriminating between various hemoplasmas has greatly enhanced diagnosis of these parasites, and has led to the identification of several new *Mycoplasma* species.

Canine Hemotropic Mycoplasmas

Two hemoplasmas, *Mycoplasma haemocanis* (previously *Haemobartonella canis*) and “*Candidatus Mycoplasma*

haematoparvum”, infect domestic canines. *M. haemocanis* causes acute hemolytic anemia in dogs that are splenectomized, immunocompromised, or have concurrent infections.³⁹ These dogs usually have sufficient circulating parasites for them to be easily demonstrated on blood films. Anemia may be severe; spherocytes may be observed and Coombs’ test results may be positive. By contrast, healthy dogs typically develop chronic, asymptomatic infections with sporadic, low-grade parasitemia.³⁹ Transmission is presumed to occur via arthropod vectors and has been experimentally demonstrated with the brown dog tick, *Rhipicephalus sanguineus*. Transmission of organisms may also occur via blood transfusion from asymptomatic carriers, but the recipient must be splenectomized or otherwise severely compromised for hemolytic disease to occur.³⁹

On routine blood films *M. haemocanis* organisms are quite pleomorphic, but have a tendency to form long chains across the surface of RBCs, sometimes with “violin bow” morphology. Individual organisms are 1–3 μm and can appear round, rod-shaped, or in ring form (Fig. 31.7).⁵⁷

“*Candidatus Mycoplasma haematoparvum*” was recently identified in a California dog with a T cell lymphoproliferative disease that had undergone splenectomy and blood transfusions.⁵¹ Molecular characterization found the organism to be most similar to “*Candidatus Mycoplasma haemominutum*” in cats. These organisms are smaller than *M. haemocanis* or *M. haemofelis* (<0.3 μm) and, therefore are more difficult to appreciate on blood films. Knowledge about the pathogenic potential of “*Candidatus M. haematoparvum*” awaits further studies.

Feline Hemotropic Mycoplasmas

Three hemoplasmas, *Mycoplasma haemofelis* (previously *Haemobartonella felis*), “*Candidatus Mycoplasma haemo-*

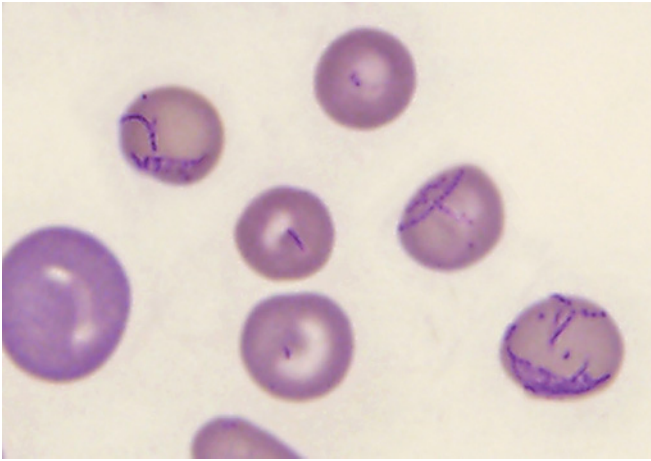


FIGURE 31.7 Blood film from a dog with *M. haemocanis* infection. Organisms are visible as chains of cocci on the surface of RBCs. Romanowsky stain; 1,000 \times .

minutum”, and “*Candidatus Mycoplasma turicensis*”, are currently known to infect domestic cats. *M. haemofelis* was previously described as the large variant or Ohio strain to differentiate it from the small variant or California strain that was later named “*Candidatus M. haemominutum*”.³⁸

Mycoplasma haemofelis causes an acute hemolytic anemia characterized by variable numbers of circulating RBC parasites. Unlike *M. haemocanis*, disease occurs in immunocompetent hosts; however, retrovirus infection (FeLV or FIV) may be a predisposing factor in some cases. Hemolysis is primarily extravascular and results in a regenerative anemia that may be severe. RBC agglutination may be present and Coombs’ test results are frequently positive. Parasitemia is cyclic, with a gradual increase in parasite numbers over a period of days followed by a sudden decline; infected RBCs may disappear from circulation in as little as 2 hours.²⁴ Cats that recover remain carriers despite treatment, with variable low-grade parasitemia.

Transmission is presumed to occur by arthropod vectors such as fleas, but experimental evidence of such transmission is lacking. Transmission has been accomplished by blood transfer. Iatrogenic transmission via blood transfusions is thus a concern, prompting recommendations for screening of potential blood donors using PCR-based DNA tests.²³ Vertical transmission from queens to kittens may also occur.

Morphology of *M. haemofelis* on routine blood films is similar to that described for *M. haemocanis*, with round (<0.8 μ m), rod (up to 1.5 μ m), and ring forms observed singly, in clusters, and short chains (Fig. 31.8).

“*Candidatus Mycoplasma haemominutum*” is reported to be about half the size of *M. haemofelis*, hindering visualization on blood films. Prevalence investigations utilizing PCR-based DNA tests indicate there is substantial subclinical infection in domestic cats, supporting the experimental evidence that “*Candidatus M. haemominutum*” is less pathogenic than *M. haemofelis*.⁵² However, there is evidence that immunosuppressed

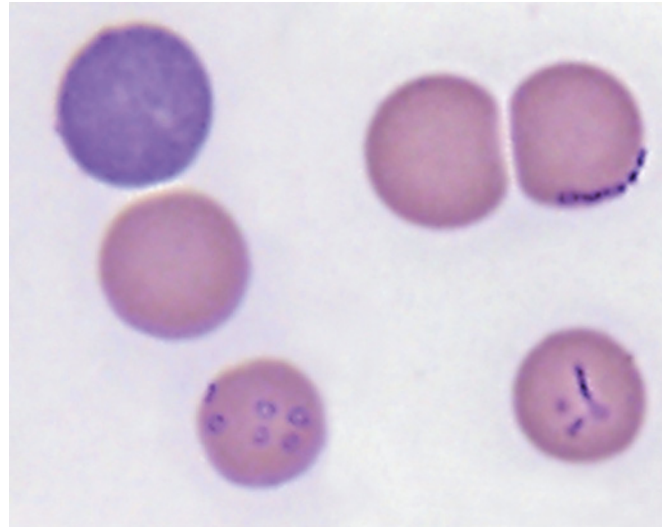


FIGURE 31.8 Blood film from a cat with *M. haemofelis* infection. Organisms are visible as chains of cocci and individual ring forms on the surface of RBCs. Romanowsky stain; 1,000 \times .

cats or those coinfecting with *M. haemofelis*, FIV, or FeLV may develop clinically significant anemia. Additionally, evidence is starting to emerge that “*Candidatus M. haemominutum*” may act as a primary pathogen in some cats, causing PCVs as low as 8%.⁴⁷

“*Candidatus Mycoplasma turicensis*” was recently identified in a Swiss cat with severe hemolytic anemia.⁵⁹ Several studies indicate this organism is relatively common, widely distributed, and frequently associated with “*Candidatus M. haemominutum*” coinfection.⁵² To date, this organism has been detected only by molecular methods. Limited experimental work suggests this organism is capable of causing severe anemia in immunocompromised cats, and mild anemia in healthy cats.⁵⁹

Swine Hemotropic Mycoplasmas

Two hemoplasmas, *Mycoplasma suis* (previously *Eperythrozoon suis*) and *Eperythrozoon parvum* are known to infect swine. *M. suis* has a worldwide distribution and causes hemolytic anemia accompanied by icterus in neonatal pigs, feeder pigs, and pregnant sows. Splenectomy of latently infected animals results in a marked increase in parasitemia, as does immunosuppressive therapy. On routine blood films *M. suis* may appear as small (approximately 1 μ m) cocci, rods, or ring forms occurring individually, in chains, or clusters. Similar to *M. haemofelis*, parasitemia is frequently transient. Newer PCR based DNA tests are proving sensitive and specific for the diagnosis of *M. suis* in the absence of demonstrable parasitemia.²⁵

Eperythrozoon parvum was originally described as coccoid or ring-shaped organisms on RBCs of splenectomized pigs.⁴⁹ This organism was approximately half the size of *M. suis*, and despite heavy parasitemia few clinical signs were observed. Recently, a hemoplasma believed to be *E. parvum* was identified in the blood of

pigs using a PCR protocol designed to detect the feline parasite "*Candidatus M. haemominutum*".⁴⁰ This organism will likely be reclassified as a mycoplasma, and renamed.

Ruminant Hemotrophic Mycoplasmas

Two hemoplasmas are currently known to infect ruminants. *Mycoplasma wenyonii* (previously *Eperythrozoon wenyonii*) infects cattle, and *Mycoplasma ovis* (previously *Eperythrozoon ovis*) infects sheep and goats. *M. wenyonii* occurs worldwide and is typically thought of as an opportunistic pathogen, resulting in clinical anemia in splenectomized or otherwise compromised cattle. Parasitemia without anemia has been reported associated with fever and mammary, scrotal, or hind limb edema.^{41,48} The mode of transmission for *M. wenyonii* is unknown, but presumed to occur via vectors such as lice, flies, ticks, and mosquitoes. Iatrogenic infection from blood transfer by contaminated needles and equipment may also occur. On blood films, *M. wenyonii* organisms are small and pleomorphic but frequently coccoid or ring-shaped, and may be found free in the background in large numbers.⁴⁸ Parasitemia is transient, however, and PCR-based DNA tests may be more sensitive than microscopy for detection of latent infections.⁵⁶

M. ovis affects sheep and goats worldwide. While frequently considered relatively non-pathogenic, the severity of hemolytic anemia with acute infection is variable depending on age, nutrition status, concurrent worm burden, and possibly *M. ovis* strain differences.³⁸ Fatal disease occurs more often in lambs than in adults. Natural transmission may occur via arthropod vectors such as mosquitoes and ticks, and iatrogenically by blood transfer. On blood films, *M. ovis* organisms are coccoid, rod, or ring shaped, 0.5–1.0 μm in diameter, and are frequently seen free in the background.

Camelid Hemotrophic Mycoplasmas

"*Candidatus Mycoplasma haemolamae*", originally described as an eperythrozoon-like parasite, affects llamas and alpacas. Anemia varies from mild to severe, with more severe clinical signs found in younger animals that are stressed or have other concurrent diseases; adults may be asymptomatic. Heavy parasitemia has been identified in crias as young as 1 day of age, suggesting vertical transmission in utero. On blood films, "*Candidatus M. haemolamae*" organisms are coccoid to ring shaped, 0.5–1.0 μm in diameter, and are often free in the background (Fig. 31.9).⁴⁶ Asymptomatic carriers have been identified using a DNA-based PCR assay.⁵³

PROTOZOA

Members of two groups of apicomplexan protozoal erythroparasites, the *Babesias* and *Theilerias*, will likely eventually undergo reorganization subsequent to genetic sequencing and phylogenetic studies.

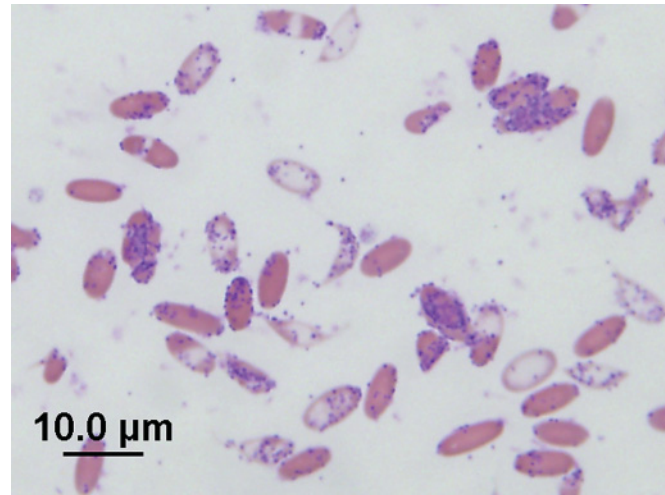


FIGURE 31.9 Blood film from a llama with "*Candidatus M. haemolamae*" infection and iron deficiency. Large numbers of coccoid organisms are present on the surface of RBCs and free in the background. Romanowsky stain.

Historically, morphology and features of their life cycles defined them. Neither genera form pigment in the parasitized cell (as opposed to *Plasmodium* and *Hemoproteus*). Both are transmitted by ixodid ticks, but transovarial tick transmission occurs with *Babesia* species and not with *Theileria* species.³⁶ Additionally, *Babesia* sporozoites directly invade erythrocytes, while *Theileria* sporozoites first invade lymphocytes or macrophages to form schizonts.³⁶

Theileriosis

Ruminants

There are multiple *Theileria* species that affect domestic and wild ruminants, primarily in Africa, Europe, Australia, and Asia. The life cycle of these parasites involves a schizont stage, typically within lymphocytes, in addition to piroplasms within RBCs.³⁶ An interesting feature of the life cycle of some *Theileria* is the reversible transformation and clonal expansion of lymphocytes that occurs as a direct result of infection.¹⁶

Theileria parva is the most economically important organism, causing the rapidly fatal bovine disease East Coast Fever in Africa.⁶ The schizont stage of *T. parva* is the major cause of pathology. Anemia is typically mild and non-regenerative, but may become severe as erythroid precursors in bone marrow are infected. *Theileria annulata* causes tropical theileriosis in cattle from the Mediterranean to Asia.⁶ Both the schizont stages and erythrocytic piroplasms are pathogenic, with severe hemolytic anemia and icterus a common occurrence. *Theileria hirci* (also known as *T. lestoquardi*) causes a similar hemolytic disease in sheep and goats in Asia and Africa. Many other *Theileria* species have been described, including *T. mutans*, *T. sergenti*, *T. orientalis* in cattle and *T. cervi* in deer and elk, but seem to be less pathogenic under most circumstances. *Theileria buffeli* has recently

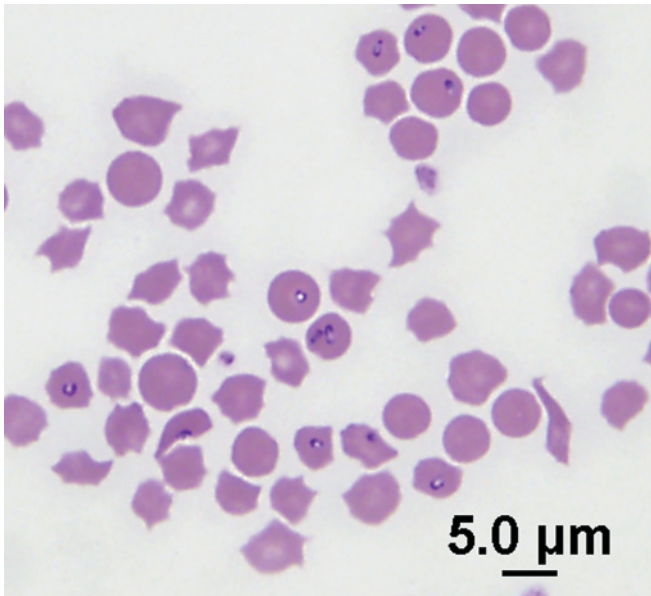


FIGURE 31.10 Blood film from a white-tailed deer with *T. cervi* infection. Small round to oval “signet-ring” piroplasms are present within RBCs. Romanowsky stain.

been recognized as a cause of hemolytic disease in cattle in the United States; however, asymptomatic infections were also detected.⁵⁰

On routine blood films theilerial piroplasms are pleomorphic, occurring as round, rod, comma, piriform, signet-ring, and racquet shapes, with occasional tetrad (Maltese-cross) formation (Fig. 31.10). The particular species cannot be determined based on morphology. Schizonts may be identified in lymphocytes in lymph node biopsies or aspirates.

Equines

The small piroplasm formerly described as *Babesia equi* has been renamed *Theileria equi*.³⁵ *T. equi* causes fever, hemolytic anemia, and icterus in horses in tropical and subtropical regions of the world. It is not yet a problem in the USA, despite the presence of appropriate tick vectors. The anemia varies from mild to severe, and subclinical infections may be detected by serology. *T. equi* piroplasms are small (1–2 μm) round to oval signet-ring structures within RBCs. Dividing stages may appear piriform in Maltese-cross arrangement.³⁵

Canines

Small signet-ring piroplasms have been identified in RBCs from Spanish dogs with fever and hemolytic anemia.⁶¹ This organism has been provisionally named *Theileria annae*. *Babesia conradae*, a small piroplasm identified in California dogs with hemolytic anemia, has been shown to be more similar to *Theileria* species than to other *Babesia* species based on genetic studies. Thus, the specific nomenclature for many of these small piroplasms will likely be revised.

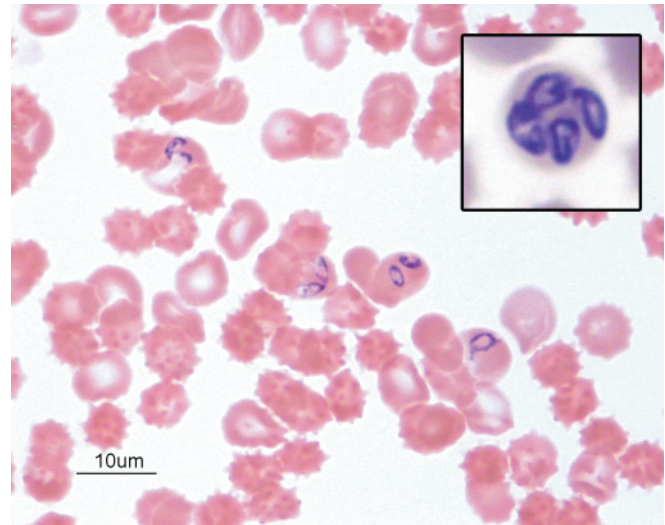


FIGURE 31.11 Blood film from a dog with *B. canis* infection. This animal did not have evidence of hemolysis (note lack of polychromasia) and probably represents a recurrent parasitemia in a chronically infected dog with subsequent immunosuppression from concurrent illness. Note the characteristic paired, piriform organism in erythrocytes. Inset: RBC containing four organisms. Romanowsky stain.

Babesiosis

In contrast to *Theileria* species, pathogens of the genus *Babesia* are limited to RBCs within the host. Sporozoites injected into the host directly attach to, penetrate, and multiply within RBCs.⁶⁰ They are classified morphologically as large (>3 μm) or small (<3 μm). Organisms exit by rupturing the host RBC and then invade new RBCs. Hematologic findings are primarily hemolytic anemia, although thrombocytopenia is common with many *Babesia* infections.

Canines

Large Canine Babesia Large canine *Babesia* has historically been called *Babesia canis*. The organism appears as large (up to 5 μm), single or paired piriform bodies (Fig. 31.11). Differences in clinical syndromes, vectors, serologic tests and cross-immunity have led to the designation of three subspecies or strains: *B. canis canis*, *B. canis vogeli* and *B. canis rossi*.

B. canis vogeli is the large *Babesia* found in the USA and also occurs in Africa, Europe, Asia, and Australia. It causes relatively mild disease and is endemic in many populations of kenneled Greyhound dogs.⁷ Infections are often subclinical with clinical disease most common in pups. Hematologic manifestations include a hemolytic anemia and thrombocytopenia. Parasites are rarely seen in the peripheral blood of subclinically affected animals and diagnosis generally is made by serology.

B. canis rossi is the most virulent strain and is found in Africa. It causes a severe, hemolytic anemia that can be both intra- and extravascular. Thrombocytopenia is also common. This parasite can induce a wide spectrum

of disease manifestations unrelated to hemolysis, referred to as “complicated” babesiosis.²⁶

B. canis canis is found in Europe and is of moderate pathogenicity. Recently a fourth, genetically distinct, large *Babesia* causing hemolytic anemia has been identified in several dogs from North Carolina.³⁰

Small Canine Babesia *Babesia gibsoni* has long been recognized as a problem of dogs in Asia, Africa, and Europe and has recently been identified as an emerging infection of dogs in the USA.⁵ In the United States, the majority of the affected dogs are American Pit Bull Terriers (APBT) or dogs that have been bitten by Pit Bulls.³¹ Acutely, the organism causes a hemolytic anemia and thrombocytopenia.^{5,37} Surviving dogs remain subclinical carriers and the incidence of such carriers is quite high in certain populations of APBTs.³¹ *B. gibsoni* can be transmitted between dogs by tick vectors, direct blood inoculation or transplacental transmission.^{20,37} In most endemic areas, *B. gibsoni* is transmitted by *Hemophysalis* ticks.⁵ It is not known if there is a competent tick vector in the United States. Clinical signs are non-specific and generally relate to hemolytic anemia. The anemia is regenerative; spherocytosis and positive direct Coombs’ test results suggest an immune-mediated component. Marked thrombocytopenia is a frequent finding and may occur before and last longer than parasitemia and/or anemia.³⁷ Parasitemia is detectable in the acute phase of infection, but is generally low. Parasites are small (1–2.5µm) and pleomorphic.³¹ The most common shape is a signet ring, although elongated forms are also seen (Fig. 31.12). Splenectomized animals show more severe clinical signs, more profound anemia, and a greater parasitemia than animals

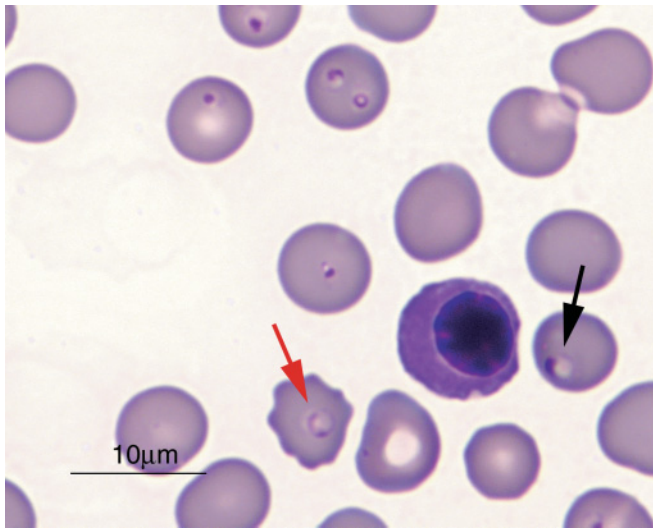


FIGURE 31.12 Blood film from an American Pit Bull Terrier with *B. gibsoni* infection. This dog suffered an acute hemolytic crisis after splenectomy for removal of a splenic tumor. Note the varying sized organisms, many of which have the classic signet-ring appearance. In some, the nuclear material is punctate (black arrow) while in others the nuclear material is spread along the periphery of the ring (red arrow). Romanowsky stain.

with an intact spleen.³⁷ Serologic tests and PCR are useful for detecting chronically infected animals and have been used in prevalence studies.⁴

A small *Babesia* causing hemolytic anemia and thrombocytopenia in dogs from California was originally assumed to be *B. gibsoni* based on morphology and host.¹³ It was later shown to be genetically distinct and was renamed *Babesia conradae*.²⁸ This organism is associated with a higher parasitemia and more profound anemia than *B. gibsoni*.

Ruminants

There are four widely recognized species of *Babesia* in cattle: *B. bovis*, *B. bigemina*, *B. divergens* and *B. major*, *B. divergens* and *B. major* are reported in cattle in Europe.⁵⁴ *B. bovis* and *B. bigemina* are the most widespread and of most economic significance. *Boophilus* and *Rhipicephalus* ticks serve as vectors, although iatrogenic transmission by direct blood transfer also occurs.

B. bigemina is a large piroplasm often appearing as the classical pair of piriform organisms. *B. bovis* is a small piroplasm frequently seen as a single round body. Both organisms cause an acute febrile illness and hemolytic episode. Calves infected at less than 8 months of age often develop subclinical disease due to innate resistance.¹⁴ Release of replicating parasites from RBCs causes intravascular hemolysis with resultant hemoglobinemia and hemoglobinuria. Increased osmotic fragility of all RBCs may result in massive terminal hemolysis despite reduced parasitemia.¹⁴ Marked accumulation of *B. bovis* parasitized RBCs in small cerebral capillaries can result in ischemia. The associated CNS signs are termed “cerebral babesiosis”.

Two species, *B. ovis* and *B. motasi*, cause disease in sheep and goats in Africa, Asia, and Europe.⁵⁴ *B. ovis* is a small oval piroplasm, while *B. motasi* is larger and is commonly seen as paired, piriform organisms.

Equines

Babesia caballi is the only true equine *Babesia* species since *B. equi* has been properly reclassified as *T. equi*. It is a large, piriform organism typically appearing in pairs with an acute angle. It is tick transmitted, and transovarial transmission may occur through several generations of ticks. The distribution of *B. caballi* includes Africa, the Americas, Asia, and Europe. Despite the presence of potential tick vectors, it is not a significant disease in the USA. Disease induced by *B. caballi* is generally much less severe than that of *T. equi*. Pronounced clinical signs and severe anemia are uncommon. Hemoglobinuria is not typical. The parasitemia is generally extremely low, even in splenectomized animals.¹⁵ Foals are immune for the first 6–9 months of life from passive immunity and innate factors.

Felines

Babesia felis is a small piroplasm reported regularly in cats from coastal regions of South Africa.⁴⁵ No vector

has been identified although it is assumed to be tick borne. It is most common in young adult cats, which may have severe regenerative anemia, hyperbilirubinemia and agglutination. The parasitemia is variable, and may be extremely high.

Feline Cytauxzoonosis

Cytauxzoon felis is a hemoparasite of wild and domestic cats that is common in the south central and southeastern USA. The disease is tick borne with bobcats, and possibly other wild felids, serving as a reservoir host.²⁷

Cytauxzoon piroplasms are found in RBCs and schizogony occurs in macrophages of various tissues. The schizogonous phase is responsible for the marked clinical disease with parasitized intravascular macrophages becoming greatly enlarged and obstructing the lumen of vessels. Clinical signs include profound depression, dehydration, marked fever and icterus. Anemia, generally non-regenerative, is a common finding that develops relatively late in the course of disease. Leukopenia and thrombocytopenia are also common. Diagnosis is usually confirmed by identification of piroplasms on peripheral blood films. The characteristic shape most easily differentiated from artifact is the signet-ring. This is a small ring (~1–3 μ m) with a round or linear thickening of nuclear material at one point of the ring (Fig. 31.13).⁵⁸ Schizont-containing macrophages can be identified in impression smears of many tissues including lung, spleen, liver, lymph nodes and bone marrow, and may also be observed on peripheral blood films late in the disease course. Parasitized macrophages become greatly enlarged and have a large, prominent nucleolus (Fig. 31.14). The prognosis for cats with cytauxzoonosis has been grave with mortality rates of nearly 100%. Survival of cats unrelated to treatment has been reported and may suggest the presence of less virulent strains.

Trypanosomiasis

Trypanosomes are flagellated protozoal organisms. They are not a significant cause of anemia in animals in North America.

Trypanasoma cruzi, etiologic agent of Chagas' disease, has been reported in dogs in several states.³ The organisms may be found in the peripheral blood in the acute stages of infection; however, anemia is not a significant finding. Organisms are fusiform, approximately 15–20 μ m in length, with a centrally located nucleus and small basophilic subterminal kinetoplast. (Fig. 31.15)

Trypanasoma theileri is a larger organism (up to 100 μ m in length) that occurs in cattle worldwide. This organism is fairly prevalent, but is considered nonpathogenic.¹⁷

Anemia in animals is caused by a variety of African trypanosomes including *T. congolense* and *T. vivax* in cattle; *T. brucei* and *T. congolense* in goats and sheep; *T. brucei* in horses and donkeys; and *T. brucei* and *T. congolense* in dogs.¹² The pathogenesis of anemia is

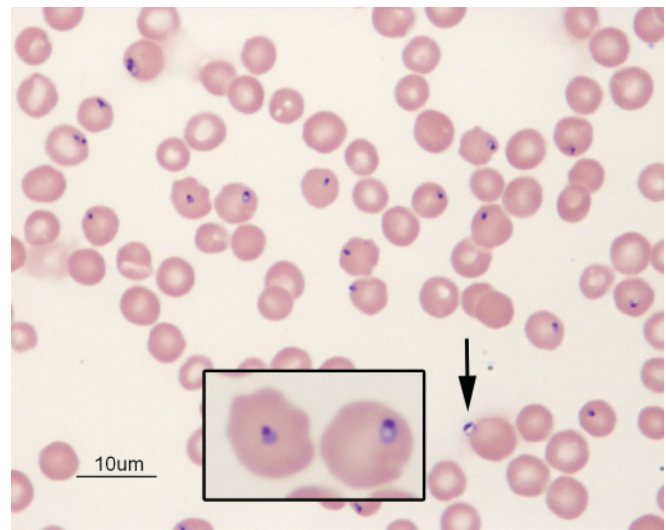


FIGURE 31.13 Blood film from a cat in with terminal cytauxzoonosis. In fatal cases, parasitemia typically progressively increases with time. Organisms are variably sized and somewhat pleomorphic. Only some demonstrate the characteristic signet-ring shape. One extracellular piroplasm is present (arrow). Inset: Higher magnification of two piroplasms. In the larger one on the right, the inner portion of the ring can be seen to be lighter than the RBC. Romanowsky stain.

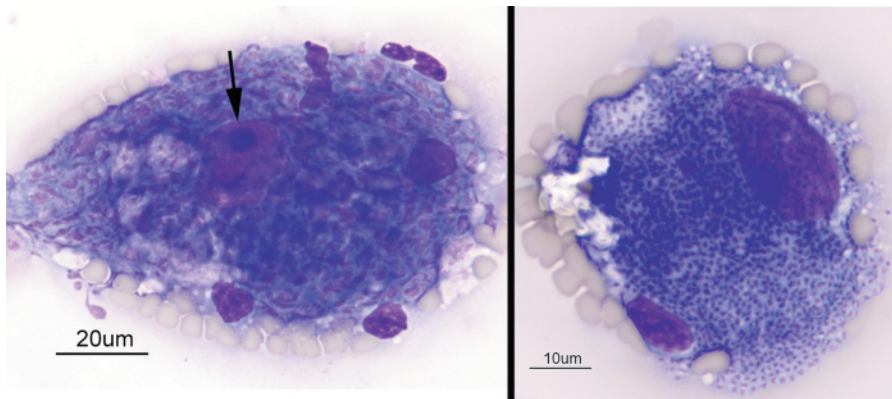


FIGURE 31.14 Large mononuclear cells with *C. felis* schizonts from the feathered edge of a blood film of a cat with cytauxzoonosis. The schizont on the left is less mature. Nuclear material is visible as variably sized, amorphous purple areas within the basophilic schizont. The schizont on the right, from the same blood film, is more mature. Nuclear material has transformed to fine punctate inclusions of developing merozoites. Romanowsky stain.

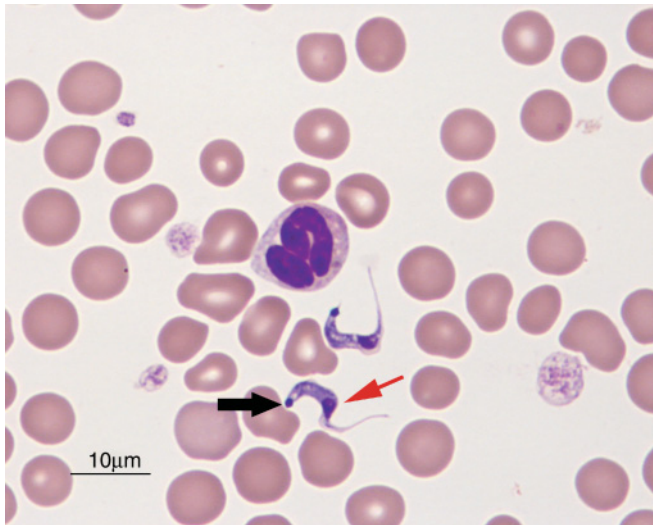


FIGURE 31.15 Blood film from a dog with *T. cruzi* infection. This dog was treated with multiple immunosuppressive agents for immune-mediated hemolytic anemia. Organisms were visible on follow-up blood films after resolution of anemia. Note the dark, subterminal kinetoplast (black arrow) and larger purple nucleus (red arrow). Romanowsky stain.

complex. In acute disease, RBC membranes are damaged directly by hemolysins released from trypanosomes. Extravascular RBC removal also occurs in the spleen, bone marrow and lungs secondary to coating of RBCs with trypanosomal antigens.¹²

Sarcocystosis

Several different species of *Sarcocystis* have been reported to cause anemia in ruminants including cattle, sheep, and goats. However, there is no parasitic stage of *Sarcocystis* that infects RBCs; thus there are no specific hematologic findings. Anemia related to sarcocystosis is thought to be due to hemolysis. In experimental infections, it reaches a nadir at about 1 month post-inoculation. Second generation meronts are maturing in endothelium at that time, suggesting that microangiopathy due to vasculitis may play a role in the anemia. Calves inoculated with *S. cruzi*, the most pathogenic species for cattle, develop a severe anemia attributed to extravascular immune-mediated hemolysis, and have evidence of a consumptive coagulopathy attributed to endothelial cell damage and disseminated intravascular coagulopathy.¹⁹ The anemia is reportedly minimally regenerative, possibly due to superimposed anemia of inflammatory disease.³³

Haemoproteus

Many different species of *Haemoproteus* can affect birds and reptiles. In general they are considered relatively non-pathogenic, although co-infection with other hemoparasites may have detrimental effects in wild populations. Clinical disease seems to be associated with the tissue stages of parasite development, rather than the

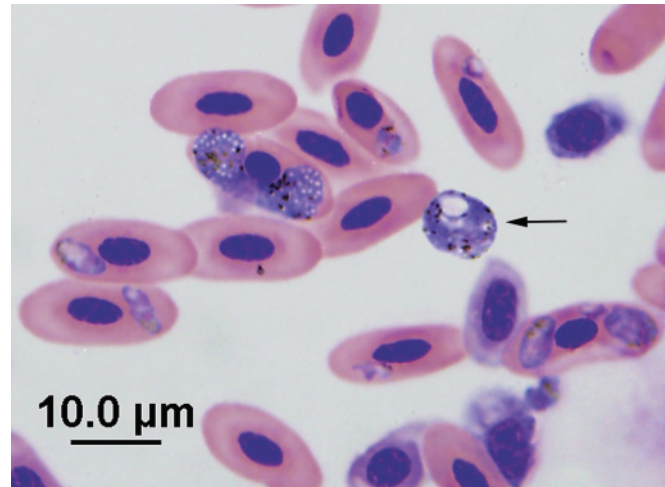


FIGURE 31.16 Blood film from an owl with *Haemoproteus* infection. Several different stages of intracellular gametocytes containing yellow-brown hemozoin pigment are visible, which surround but do not displace the nucleus. One extracellular form is present (arrow). Wright's stain.

RBC stages. Anemia may develop in parasitized animals that are ill, injured, or immunocompromised, with a few reports of mortality associated with anemia occurring in young quail and pigeons. Parasitemia is frequently observed in raptors undergoing rehabilitation for illness or injury, and the magnitude of parasitemia frequently decreases as birds recover.⁴⁴

Haemoproteus gametocytes are generally present in RBCs, although extra-erythrocytic forms may occasionally be seen on blood films (Fig. 31.16). Gametocytes vary in appearance depending on their stage of development, and may be round, oval, or elongated.^{10,34} Large forms typically encircle the RBC nucleus without displacing it. Gametocytes usually contain refractile yellow to brown iron pigment (hemozoin), a result of hemo-globin digestion (“malarial pigment”).

Plasmodium

Parasites of the genus *Plasmodium* are the cause of malaria in humans and many animal species. Numerous different *Plasmodium* species have been identified. In veterinary medicine, malarial parasites are most often encountered in birds and reptiles. Some species of birds seem more susceptible to clinical disease (canaries, penguins, ducks, pigeons, raptors, domestic poultry), while others may be asymptomatic carriers. Reported disease manifestations include hemolytic anemia, leukocytosis, lymphocytosis, hepatomegaly, splenomegaly, and death.¹⁰

Gametocytes contain yellow-brown iron pigment (hemozoin), similar to that found in *Haemoproteus* gametocytes. Gametocytes may be elongated or round depending on the particular species; round forms tend to displace the host cell nucleus, unlike *Haemoproteus* (Fig. 31.17). Schizonts may be found in peripheral blood RBCs, thrombocytes, and leukocytes. They have

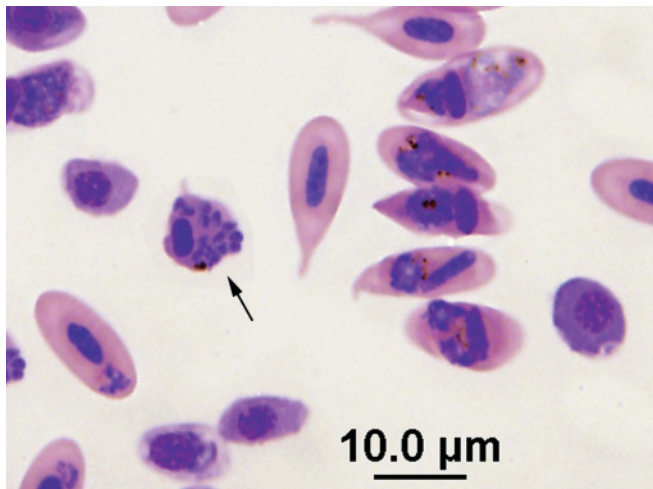


FIGURE 31.17 Blood film from a golden eagle with *Plasmodium* infection. Developing gametocytes containing yellow-brown hemozoin pigment displace RBC nuclei. One erythrocyte contains developing merozoites (arrow). Wright's stain.

a variable appearance depending upon the stages of the developing merozoites, which may appear fused or as distinct basophilic structures. Trophozoites are round to oval with an eccentric nucleus, resulting in a signet-ring appearance. Features of *Plasmodium* useful to differentiate it from *Haemoproteus* are presence of schizonts in peripheral blood, developing parasite stages within leukocytes and thrombocytes in addition to RBCs, and displacement of the host cell nucleus.¹⁰ However, there are significant differences in the appearance of these parasites in blood cells; depending upon the particular *Plasmodium* species observed, schizonts may only be observed within RBCs, and the host cell nucleus may or may not be displaced. Those interested in detailed morphologic descriptions of avian haemosporidia are referred to the comprehensive monograph by Valkiunas.⁵⁵ Recently developed DNA-based PCR assays should prove helpful to better understand and classify the various morphologic variants.

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Anemia Associated with Bacteria and Viral Infections

CASEY M. RIEGEL and STEVEN L. STOCKHAM

Yellow Lamb Disease by (Enterotoxemic Jaundice, Yellows) Caused by *Clostridium perfringens* type A
Bacillary Hemoglobinuria, Red Water Disease, or Nevada Red Water Caused by *Clostridium haemolyticum* (or *C. novyi* type D)

Corynebacteriosis Caused by *Corynebacterium pseudotuberculosis*
Leptospirosis Caused by *Leptospira interrogans* serovars *pomona* and *icterohaemorrhagiae*
Equine Infectious Anemia Caused by Equine Infectious Anemia Virus

Acronyms and Abbreviations

AGID, agar gel immunodiffusion; CBC, complete blood count; CELISA, competitive enzyme-linked immunosorbent assay; EIA, equine infectious anemia; ELAV, equine infectious anemia virus; ELISA, enzyme-linked immunosorbent assay; IgM, immunoglobulin M; RBC, red blood cell; WBC, white blood cell.

Many bacterial and viral infections can directly or indirectly cause anemia. This chapter's primary topics are the anemias associated with infections by *Clostridium*, *Corynebacterium*, *Leptospira*, and equine infectious anemia virus (Table 32.1). Anemias caused by feline viruses (i.e. feline leukemia virus and feline immunodeficiency virus), rickettsial bacteria (e.g. *Rickettsia*, *Ehrlichia*, *Anaplasma*) or mycoplasmal bacteria (e.g. *Mycoplasma haemocanis*, *Mycoplasma haemofelis*) are described in Chapters 19, 31, 55, and 62, respectively.

Most anemias associated with bacterial and viral infections are caused by the inflammatory reaction (i.e. anemia of inflammatory disease, see Chapter 37). Infections may cause more severe anemias if they result in hemorrhage (vascular damage, thrombocytopenia), red blood cell (RBC) fragmentation (vasculopathy, coagulopathy), or generalized bone marrow damage (marrow necrosis or replacement, see Chapters 17 and 18).

Infectious agents, their products, or the animal's specific response to the agent may cause an anemia via three major processes: (1) damaged circulating RBCs causing intravascular or extravascular hemolysis, (2) damaged erythroid precursors causing erythroid hypoplasia, (3) damaged hematopoietic or supportive stromal cells causing general marrow hypoplasia.

YELLOW LAMB DISEASE (ENTEROTOXEMIC JAUNDICE, YELLOWS) CAUSED BY *CLOSTRIDIUM PERFRINGENS* TYPE A (BASYNYM *CLOSTRIDIUM WELCHII*)

Yellow lamb disease occurs in spring nursing lambs of northern California and Oregon²² and in lambs and calves in Canada, Australia, New Zealand, and South Africa.²⁸ *Clostridium perfringens* infections in two horses were associated with intravascular hemolytic anemias that were considered immune-mediated.⁵⁸ Hemolytic anemias are not described in most enteric diseases caused by *C. perfringens* type A.

Clinical signs include depression, fever, weakness, pale mucous membranes, anemia, hemoglobinuria, icterus, and diarrhea. Death may occur within 6–12 hours of onset of illness especially if icterus and hemoglobinuria are present.^{22,28} Changes in blood are consistent with massive intravascular hemolysis.^{6,28,38}

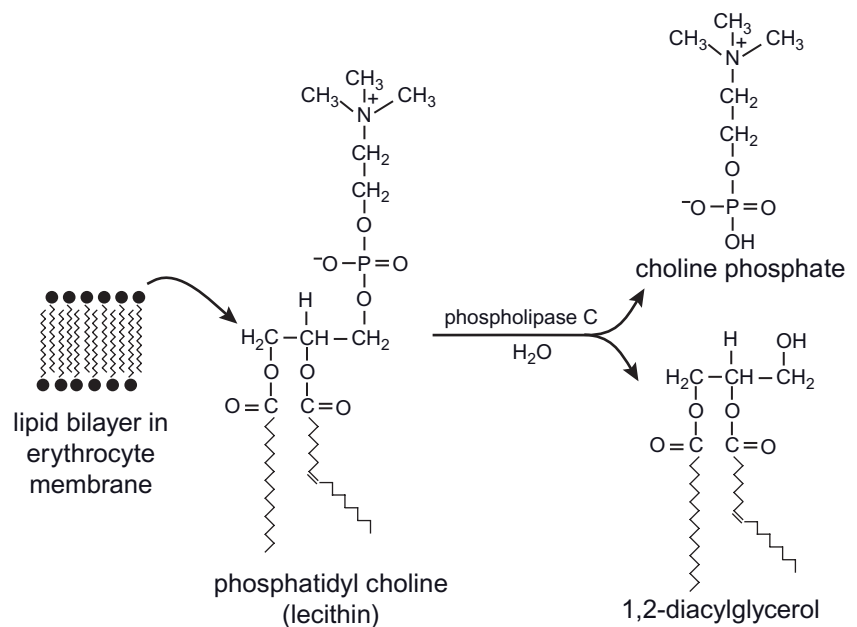
An α toxin, a phospholipase C, which is produced by *C. perfringens* type A, causes the RBC damage.²⁰ The α toxin causes hydrolysis of membrane phospholipids (Fig. 32.1) and resultant lysis of red blood cells (RBCs), white blood cells (WBCs), platelets, endothelial cells, and myocytes.³³

TABLE 32.1 Bacterial and Viral Infections that Cause Anemia^a

Organism	Disease	Susceptible Animals
<i>Clostridium perfringens</i> type A	Yellow lamb disease, enterotoxemic jaundice, or yellows	Lambs, calves, captive Siberian ibex, horses
<i>Clostridium haemolyticum</i> (or <i>C. novyi</i> type D)	Bacillary hemoglobinuria, red water disease, or Nevada Red Water	Cattle, sheep
<i>Corynebacterium pseudotuberculosis</i>	(Experimental infection)	Sheep
<i>Leptospira interrogans</i> serogroups	Leptospirosis	Calves, lambs, pigs, perhaps rhinoceros, possibly dogs
Equine infectious anemia virus	Equine infectious anemia, swamp fever, equine malarial fever, mountain fever, slow fever	Horses, ponies, donkeys, mules

^aFeline leukemia virus, feline immunodeficiency virus, rickettsial bacteria (e.g. *Rickettsia*, *Ehrlichia*, *Anaplasma*), and mycoplasmal bacteria (*Mycoplasma haemocanis*, *Mycoplasma haemofelis*) not included.

FIGURE 32.1 Action of phospholipase C on the RBC membrane. Phospholipase C catalyzes the hydrolysis of two RBC membrane lipids – phosphatidylcholine and sphingomyelin – that are the predominant phospholipids in the outer hemileaflet of the RBC membrane. The reaction shows hydrolysis of phosphatidylcholine into its hydrophilic portion (choline phosphate) and hydrophobic portion (1,2-diacylglycerol). In a similar reaction, phospholipase C catalyzes the hydrolysis of sphingomyelin to ceramide and sphingosine 1-phosphate (not shown).



Clostridium perfringens type A is a part of intestinal microbiota in animals, but increased bacterial growth has been associated with highly proteinaceous diets,³⁶ overfeeding,³⁴ and starchy foods.³¹ After production by the bacteria, α toxin is absorbed by intestinal mucosa, enters the blood, and damages cell membranes. Major postmortem findings include evidence of massive intravascular hemolysis, renal hemoglobin casts, destruction of capillary walls leading to intestinal mucosal necrosis, centrilobular hepatic necrosis, and serosal, petechial and ecchymotic hemorrhages. In calves, abomasal hemorrhage and ulceration may be present. Small intestine may be hyperemic but rapid autolysis may obliterate the lesions; large, Gram-positive bacilli may be found in the mucosa and submucosa.²⁷

A diagnosis of yellow lamb disease is confirmed by detecting α toxin in intestinal contents or blood from fresh carcasses via serum-neutralization tests in mice

and guinea pigs.⁵ Polymerase chain reaction assays can detect the α toxin gene (also known as *cpa* gene) of *C. perfringens* type A.¹⁰

BACILLARY HEMOGLOBINURIA, RED WATER DISEASE, OR NEVADA RED WATER CAUSED BY *CLOSTRIDIUM HAEMOLYTICUM* (OR *C. NOVYI* TYPE D)

Bacillary hemoglobinuria is a fatal disease that occurs naturally in cattle and experimentally in sheep. It can be a seasonal disease (summer and early fall) and is associated with liver fluke migration. It is endemic in poorly drained or swampy areas of the Gulf coast states, western United States, South America, Wales, eastern Europe, and New Zealand.^{34,36}

Bacillary hemoglobinuria is rarely recognized antemortem because the severe hemolysis causes acute death. When seen, clinical signs include malaise, reluctance to move, arched back, bloody diarrhea, fever, rapid and shallow breathing, and blood-tinged froth from nostrils. The classic port-wine urine of hemoglobinuria is uncommon.³² If the affected animal survives the initial crisis, icterus and a regenerative anemia can develop.⁴ In experimental infections, severe anemia and death is seen within 3 days of inoculation. Calves and ewes develop fever, a gradual to precipitous drop in hematocrit values, mild to moderate leukocytosis, and increased aspartate transaminase activity (either due to RBC or hepatocyte damage).²⁵

RBC damage is caused primarily by a β toxin, a phospholipase C (lecithinase), that hydrolyzes phosphatidylcholine and sphingomyelin (Fig. 32.1). The β toxin is not related serologically to the phospholipase of other clostridial species.³⁰ The β toxin is produced by two clostridial organisms, *C. haemolyticum* and *C. novyi* type D. Other minor hemolysins are reported as products of *C. haemolyticum*.²¹

After spores are ingested, organisms cross the intestinal mucosa and are transported to liver and other organs in macrophages. Spores may persist in Kupffer cells for a long time. Anaerobic conditions, such as those induced by hepatic biopsy and the presence of liver parasites stimulate growth of organisms and production of toxins.²⁵ Absorption of β toxin into blood leads rapidly to intravascular hemolysis, damaged endothelial cells, icterus, hemoglobinuria, and death.³²

Necropsy findings may include pale and icteric mucous membranes, petechial and ecchymotic subcutaneous hemorrhages, copious amounts of red-tinged abdominal and thoracic fluid, hemoglobinuria, renal hemoglobin casts, congested or hemorrhagic lymph nodes, extensive edema, and bloody intestinal contents. Characteristic liver lesions are foci of hepatic necrosis surrounded by a reddened reaction zone.³⁴

Diagnosis is usually made from the combination of a sudden death and postmortem findings. Numerous Gram-positive bacilli may be found in liver, spleen, blood, and abdominal fluid but their presence must be interpreted with caution. Fluorescent antibody tests for detection of the organism in fresh liver tissue can be performed.¹⁷ In addition, phospholipase C activity (β toxin) can be detected in tissues.

CORYNEBACTERIOSIS CAUSED BY CORYNEBACTERIUM PSEUDOTUBERCULOSIS

Corynebacterium pseudotuberculosis produces an exotoxin that has sphingomyelin-specific phospholipase D activity. When given intravenously in sheep, the exotoxin causes an acute, severe intravascular hemolytic anemia.¹⁴ The exotoxin causes alterations in the phospholipid composition of RBC membranes leading to spherostomatocyte formation and pitting of RBC membranes.² *C. pseudotuberculosis* causes caseous lymphadenitis in

sheep and goats and other forms of abscessation; anemia in these animals is probably the anemia of inflammatory disease. Natural infections with *C. pseudotuberculosis* may not cause a hemolytic anemia due to the production of anti-toxin against the phospholipase D during early stages of the disease.

LEPTOSPIROSIS CAUSED BY LEPTOSPIRA INTERROGANS SEROVARS POMONA AND ICTEROHAEMORRHAGIAE

Leptospirae are saprophytic spirochetes that live in moist habitats throughout the world and infect humans, most domestic animals, and many wild animals.¹⁶ However, in domestic animals, the hemolytic state of leptospirosis is seen primarily in calves, lambs, and pigs.^{3,35} A leptospiral infection may be a cause of acute hemolytic anemias of black rhinoceros.¹³ Many serovars of leptospira are reported to cause anemia in dogs, but the mechanism for the anemia is not understood.¹⁵

When the hemolytic state is present, the clinical signs and clinicopathologic abnormalities can include fever, depression, anorexia, anemia, hemoglobinemia, hemoglobinuria, icterus, neutrophilia, and petechial or ecchymotic hemorrhages. In experimental infections in calves, RBC fragility was increased.²⁶ Several explanations for the hemolytic process have been proposed; experimental evidence in lambs indicates involvement of an immunoglobulin M (IgM) cold agglutinin.^{1,12}

In lambs, major necropsy lesions include hemoglobinuria, icteric membranes and tissues, severe renal tubular necrosis with casts, and periacinar hepatocellular necrosis.¹¹ A hemolytic anemia caused by leptospirosis is confirmed by finding leptospiral spirochetes in urine or other fluids by direct dark-field microscopic examination, culture of organisms with specific media, animal inoculation, and by serologic testing. Either enzyme-linked immunosorbent assay (ELISA) or agglutination tests can detect IgM agglutinins within 7–9 days of onset of disease.³⁵ Diagnosis should be based on finding at least a four-fold increase in titer to either *pomona* or *icterohaemorrhagiae* serogroups over the course of infection.

EQUINE INFECTIOUS ANEMIA (EIA) CAUSED BY THE EIA VIRUS (EIAV)

Equine infectious anemia virus occurs worldwide but infections are more prevalent in lowland swamps; thus the common name of swamp fever (see Chapters 19 and 35). The EIAV infection can produce clinical illness in horses, ponies, donkeys, and mules.

The EIAV enters the horse via insect vectors or fomites and infects cells of the mononuclear phagocyte system. The persistent viral replication in macrophages and the animal's response to the infection creates the clinical manifestations that have been put into four stages (Table 32.2).¹⁸ When clinically ill, CBC results may detect anemia, thrombocytopenia, and either a

TABLE 32.2 Stages of Equine Infectious Anemia Infection

Stage	Clinical Signs
Acute	Acute illness may last 3–5 days, sudden fever, weakness, anemia, tachypnea, anorexia, petechial hemorrhages, thrombocytopenia, dependent edema
Subacute	Febrile episodes of several days' duration occur almost monthly ^a
Chronic	Febrile episodes at intervals of 5 months or longer; weight loss, hypergammaglobulinemia ^a
Carrier	Asymptomatic

^aSee clinical signs of acute stage when febrile.

neutropenia or neutrophilia. In the acute stage, thrombocytopenia is the most consistent hematologic abnormality and is due to both decreased platelet survival and production.⁸ Either binding of complement or immune-complexes to circulating platelets causes their removal from blood by macrophages.²⁹ Also, concentrations of tumor necrosis factor- α , transforming growth factor- β , and interferon- α increase before the onset of the EIA thrombocytopenia, and these cytokines can suppress megakaryocytopoiesis.³⁷

The EIAV infection causes anemia by decreasing RBC lifespan and decreasing RBC production.⁷ During an acute stage, a horse's anemia may be accompanied by a hemoglobinemia reflecting intravascular hemolysis. The association of EIAV envelope glycoprotein with RBC membranes activates complement and causes a C3-fraction coating of RBCs and resultant hemolysis.⁹ Antibody binding may be necessary for the coating of RBCs with C3;²⁴ infected horses may be Coombs'-positive. Morphologic changes in RBCs may include anisocytosis due to macrocytosis, Heinz bodies,²³ spherocytes (difficult to detect in equine blood), and sideroleukocytes (Fig. 32.2). The pathogenesis of the progressive non-regenerative anemia of chronic EIA is similar to the anemia of inflammatory disease (see Chapter 37).

If a horse dies during the acute stage, findings may include hemorrhages in a variety of tissues, splenomegaly, enlarged lymph nodes, subcutaneous edema, and evidence of anemia. Bone marrow and lymph node hyperplasia, hepatomegaly with hemosiderosis and necrosis, and a proliferative glomerulonephritis are reported in chronically infected horses.

The diagnosis of EIA is confirmed by finding serologic evidence of the EIAV infection. The standard test has been an agar gel immunodiffusion (AGID) assay (Coggins test) that detects precipitating antibody against the EIAV's group-specific core protein, p26. A horse may produce detectable EIAV antibodies within 12–25 days post-inoculation.¹⁹ A competitive enzyme-linked immunosorbent assay (CELISA) is also available. Immunoblot assays for antibodies against three major EIAV antigens can be used to clarify discrepancies in either AGID or CELISA results.¹⁹

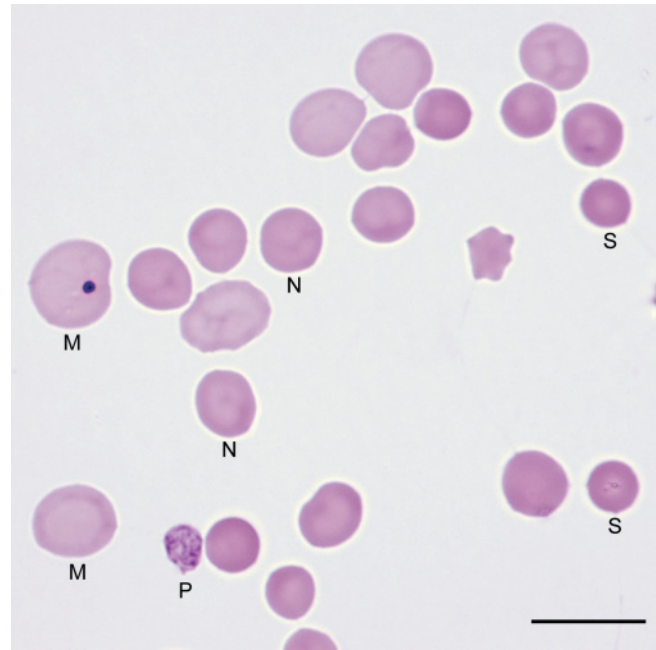


FIGURE 32.2 Blood film from a horse with acute hemolytic anemia due to EIA. The blood film contains macrocytes (M) (one contains a Howell-Jolly body), two possible spherocytes (S), normocytes (N); and a platelet (P). Modified Wright stain; bar, 10 μ m.

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Immune-Mediated Anemias in the Dog

MICHAEL J. DAY

Definition and Pathogenesis

Definitions

Immunopathogenesis of AIHA

Factors Predisposing to Canine AIHA

Immunopathogenesis of Canine AIHA

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Clinical Presentation

Diagnosis

Therapy

Outcome

Acronyms and Abbreviations

AIHA, autoimmune hemolytic anemia; ANA, antinuclear antibody; APC, antigen-presenting cell; C3, third component of complement; CD, cluster of differentiation; DAT, direct antiglobulin test; DEA, dog erythrocyte antigen; DELAT, direct enzyme-linked antiglobulin test; DIC, disseminated intravascular coagulation; Ig, immunoglobulin; IL, interleukin; IMHA, immune-mediated hemolytic anemia; IMN, immune-mediated neutropenia; IMT, immune-mediated thrombocytopenia; MHC, major histocompatibility complex; NOD, non-obese diabetic mouse; NOD, non-obese diabetic mouse; NOD, non-obese diabetic mouse; NOD, non-obese diabetic mouse; NZB, New Zealand black mouse; PCV, packed cell volume; PRCA, pure red cell aplasia; RBC, red blood cell; SLE, systemic lupus erythematosus; TNF, tumor necrosis factor.

DEFINITION AND PATHOGENESIS

Definitions

An immune-mediated hemolytic anemia (IMHA) arises when red blood cells (RBCs) (or bone marrow erythroid precursors) are destroyed via the mechanism of type II hypersensitivity, following the attachment of immunoglobulin to the cell membrane (Fig. 33.1). Antibody binding may activate the classical pathway of the complement system and result in deposition of complement components on the erythrocyte surface. If complement activation proceeds through the terminal pathway to the formation of transmembrane channels (membrane attack complexes), the RBC may be destroyed by osmotic lysis in the circulation (intravascular hemolysis). Alternatively, the surface immunoglobulin and complement may interact with Fc and complement receptors expressed by phagocytic cells (chiefly macrophages), resulting in damage to, or removal of, coated erythrocytes in extravascular sites such as the spleen or liver (extravascular hemolysis).

The terminology describing these effects must be used with care. An IMHA may be primary or second-

ary in nature. Secondary IMHA occurs when there is an underlying reason for the attachment of immunoglobulin to RBCs. For example, IMHA may occur as a secondary phenomenon in neoplastic disease or when antibody has specificity for an infectious agent or drug that is associated with the RBC surface. In these latter cases, the RBC destruction is due to “bystander hemolysis” as the causative antibody is not specific for the RBC itself.

By contrast, in primary idiopathic IMHA there is no underlying disease or evidence of recent drug or vaccine administration, and the antibody is considered to be a true autoantibody with specificity for a self-antigen of the RBC membrane.¹⁶ Only this form of disease is true autoimmune hemolytic anemia (AIHA); thus, the terms IMHA and AIHA should not be used interchangeably. AIHA may occur as a single clinical entity, or may be recognized concurrently with primary autoimmune immune-mediated thrombocytopenia (IMT; the combined disease is Evans’ syndrome), primary autoimmune immune-mediated neutropenia (IMN) or be part of the multisystemic autoimmune disease systemic lupus erythematosus (SLE). In some cases, the autoimmune response is directed against bone marrow

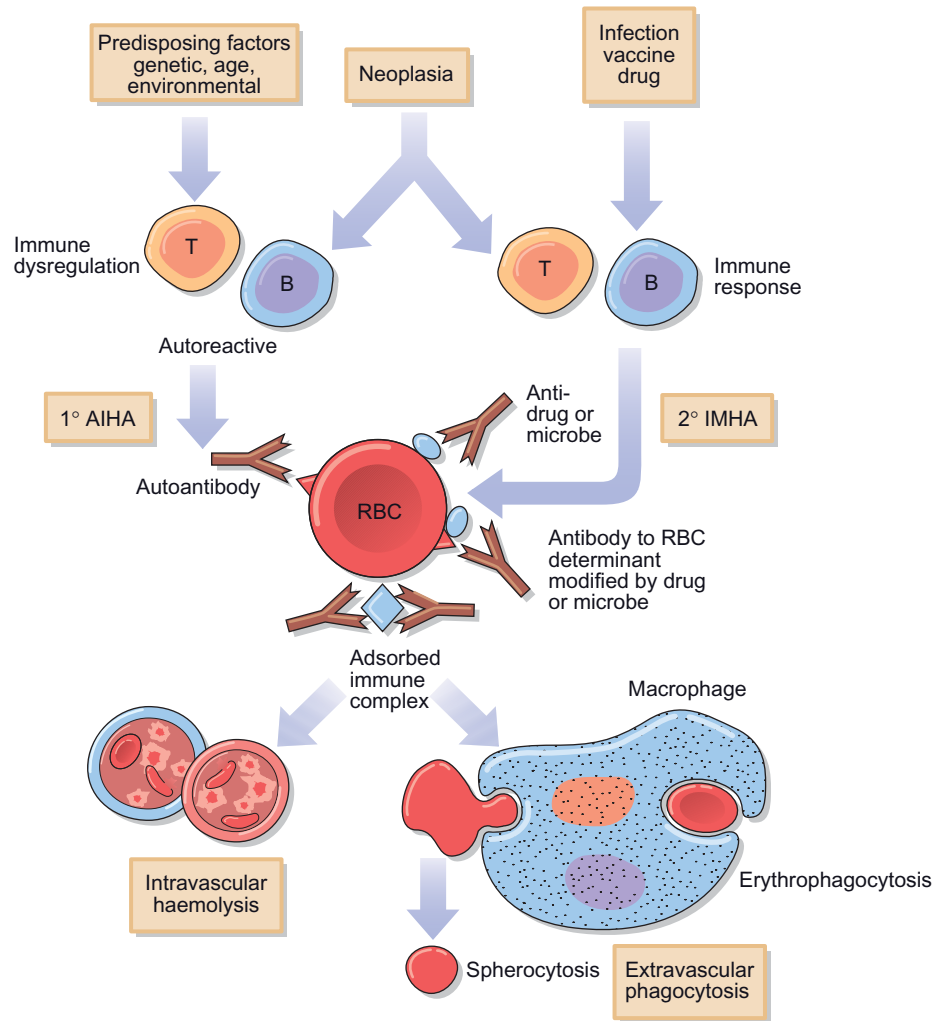


FIGURE 33.1 Pathogenesis of immune-mediated hemolytic anemia. Immune-mediated hemolytic anemia (IMHA) may be primary (autoimmune, AIHA) or secondary to a defined underlying cause. In AIHA, a series of predisposing factors interact to allow activation of self-reactive T and B cells and production of autoantibody specific for an antigen of the RBC membrane. In secondary IMHA, the erythrocyte becomes an “innocent bystander” in an immune response that is directed towards infectious agent or drug associated with the RBC membrane, or that generates immune complexes that non-specifically adsorb to the RBC surface. In either case, the RBC may be destroyed following complement fixation and formation of the “membrane attack complex” (intravascular hemolysis), or interact with phagocytic cells that express surface Fc and complement receptors. In this case the RBC may be entirely removed by erythrophagocytosis, or suffer partial loss of its cell membrane to form a spherocyte. These processes occur in the spleen or liver (extravascular hemolysis).

erythroid precursors resulting in the spectrum of change that includes both nonregenerative IMHA (NRIMHA) and acquired pure red cell aplasia (PRCA). In this group of diseases, there may or may not be autoantibodies that react with circulating RBCs in addition to the precursor population. In AIHA or NRIMHA/PRCA, erythrocyte-specific autoantibody is thought rarely to be found free in the circulation as most is bound to RBCs or precursors. The reactions to blood group antigens (blood transfusion reactions, neonatal isoerythrolysis) and the uncommon cold agglutinin disease may be considered as forms of IMHA, but these disorders are not considered in this chapter.

IMHA is one of the most common immune-mediated disorders of the dog and the majority of cases (at least in North America, Northern Europe and Australia) are currently considered to be primary AIHA.

Immunopathogenesis of AIHA

Factors underlying the development of a true autoimmune response are complex, and even now, not entirely understood. Briefly, the expression of any autoimmune disease requires that a combination of predisposing factors permits the development of an immunological alteration resulting in the observed autoimmune pathology. Such immunological alterations have been experimentally investigated using murine models of AIHA. For example, AIHA may be induced in particular inbred strains of mice that are immunized with rat RBCs,¹² or that are transgenic for high expression of interleukin (IL)-4,²² or expression of an RBC autoantibody.⁴² IL-2 deficient mice, created by targeted disruption of the IL-2 gene, develop a lymphoproliferative syndrome with multisystemic autoimmunity including Coombs’

positive hemolytic anemia.⁴⁷ New Zealand black (NZB) mice spontaneously develop autoimmunity including AIHA or a SLE-like disease characterized by immune complex glomerulonephritis and serum antinuclear antibody (ANA). The AIHA is mediated by CD4⁺ Th1 lymphocytes that are first activated in very young mice before the appearance of autoantibodies and anemia.⁵¹ A strain of non-obese diabetic (NOD) mice that develop autoimmune destruction of pancreatic beta cells mediated by T cells, culminating in insulin-dependent diabetes mellitus, may also develop Coombs'-positive hemolytic anemia late in life. However, the immune mediated anemia in the NOD strain may occur in both diabetic and non-diabetic mice.⁴

Activation of autoreactive lymphocytes is generally considered to reflect a failure of natural regulation (suppression) of such cells. A variety of mechanisms is involved in the control of self-reactive lymphocytes including: (1) central deletion of T cells with such specificity as part of intra-thymic development and of autoreactive B cells during development in the bone marrow, (2) peripheral deletion of self-reactive T cells upon exposure to autoantigenic peptide outside of the thymus, (3) the induction of anergy by exposure to autoantigen in the absence of delivery of co-stimulatory signals, (4) immunological ignorance due to failure to present autoantigen to the autoreactive populations, and (5) active suppression by regulatory lymphocytes. Particular advances have been made recently with respect to understanding of the last component. A population of "natural suppressor" T cells defined by expression of CD4 and CD25 is thought to be constitutively present within the immune system in order to prevent the activation of pathogenic T cells specific for autoantigen or allergen. These "Treg" cells are further defined by expression of specific genes (e.g. Foxp3, GITR) and although they secrete the immunoregulatory cytokine IL-10 are thought to require direct contact ("cognate interaction") with the target cells they intend to regulate. Treg cells have now been identified in the dog⁷ and it is likely that suboptimal function of this population occurs in canine autoimmunity. Other T cell types also are known to have suppressive function. Overall, T regulatory cells are involved in dampening an immune response after they have successfully tackled invading organisms as well as keeping in check immune responses that may potentially attack one's own tissues.

It is now also believed that microbial infection is of particular importance in the induction of autoimmune diseases. Clinically normal individuals have circulating lymphocytes that are programmed to recognise self-antigens, but these cells are normally incapable of responding to autoantigens (self-tolerance). The altered immunoregulation that follows infection may permit loss of self-tolerance and the subsequent expression of autoimmune disease. For example, mice infected with a particular substrain of the lymphocytic choriomeningitis virus develop transient AIHA. In this instance the anemia is not caused by antibodies that react with a shared epitope on the virus and RBC, but by true eryth-

rocyte-specific autoantibodies.³⁶ The induction of this autoimmune response is thought to be due to inappropriate activation of autoreactive T cells by virally-derived peptides that are "molecular mimics" of erythrocyte-derived peptides; these peptides would normally not be presented by the antigen presenting cells (APCs) of the immune system and thus, autoreactive T cells are maintained in a state of "immunological ignorance".

Factors Predisposing to Canine AIHA

A number of factors predisposing to the development of AIHA are defined in the dog. There is a strong genetic influence suggested by the greater prevalence of the disease in particular breeds (e.g. Old English Sheepdog, Cocker Spaniel, Border Collie, Poodle, English Springer Spaniel, Irish Setter, Miniature Schnauzer) and within particular pedigrees.^{13,18,19,58} Recent investigations have revealed associations with allotypes and haplotypes of genes of the major histocompatibility complex (MHC), which are strongest when specific breed groups (e.g. Cocker and English Springer Spaniels) are considered.³¹ Some MHC haplotypes increase susceptibility to IMHA, whereas others appear to confer protection. Current studies are investigating broader genomic associations in dogs of these breeds (LJ Kennedy and MJ Day, unpublished data). One report has suggested that expression of the blood group antigen DEA7 in Cocker Spaniel dogs reduces susceptibility to IMHA.⁴⁰

Canine AIHA is generally a disease of middle age (6–8 years). Although there is no clear gender predisposition, the disease can be precipitated in bitches by the stress of whelping or oestrus. A seasonal incidence also has been proposed for canine AIHA; however, this is not consistent between different studies and may in fact be related to the prevalence of underlying infections, or timing of vaccinations (Fig. 33.2).^{21,32,58}

Immunopathogenesis of Canine AIHA

A series of investigations has revealed many parallels between canine AIHA and the disease in humans and experimental rodent models. The autoantibodies that characterize the canine disease are heterogeneous in their class and specificity, suggesting that a range of different underlying mechanisms may be involved in triggering the disease. Both IgM and IgG autoantibodies are found, and particular subclasses (IgG1 and IgG4) dominate the IgG response.¹⁴ Significant quantities of IgA may also be associated with the erythrocyte membrane in AIHA, but the presence of this immunoglobulin is of questionable relevance. The specificity of the IgG autoantibodies has been characterized by eluting them from the surface of patient RBCs and incubating them with biotin-labeled normal canine RBC in the technique of immunoprecipitation.^{2,15} These autoantibodies are directed against various components including erythrocyte membrane glycoporphins, the anion exchange molecule (band 3) and the cytoskeletal molecule spectrin (Fig. 33.3).

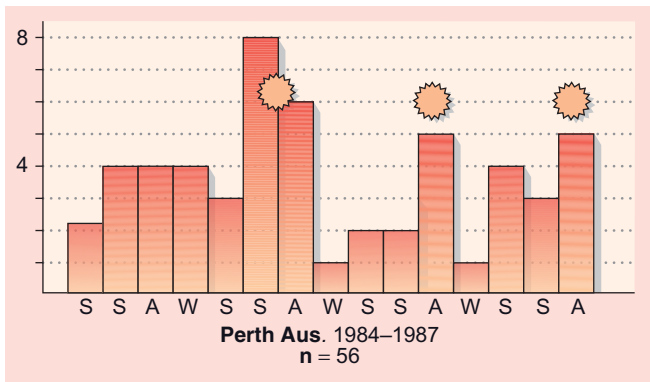
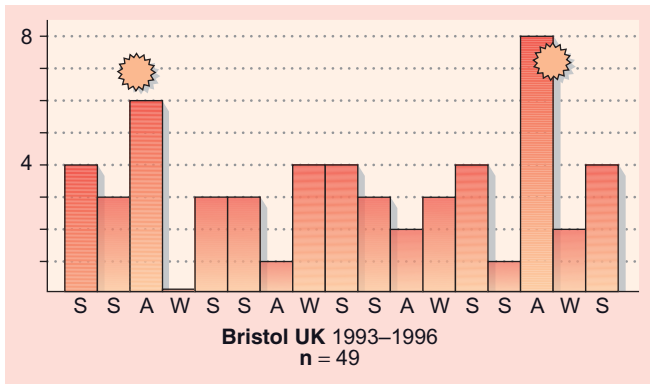


FIGURE 33.2 Seasonal distribution of cases of canine IMHA. Data are presented from the Northern (United Kingdom) and Southern (Australia) Hemispheres. The number of cases in each season (Spring, Summer, Autumn, Winter; SSAW) over a 3-year period are shown. Although there is a spread of cases, peaks (stars) are noted in some autumn periods and one Southern Hemisphere summer. Interpretation of such data is difficult, but may reflect seasonal infections or annual cycles of administration of vaccines.

T cell reactivity in canine AIHA has also been examined. Like other species, clinically normal dogs harbour RBC-reactive lymphocytes that can be induced to proliferate in vitro when challenged with RBC-derived antigens. Such cells have a greater degree of reactivity when they are obtained from dogs recovered from AIHA (memory lymphocytes) or from normal dogs that are closely related to AIHA cases.¹¹ The latter observation suggests an immunological mechanism for genetic susceptibility to AIHA in dogs. It is important to further investigate the fine specificity of such autoimmune responses, as this knowledge will form the basis for developing novel immunotherapeutic agents in future years.

Secondary IMHA in the Dog

There are numerous triggers for secondary IMHA in dogs. Before considering a diagnosis of AIHA, attempts should be made to rule out underlying disorders that are known to precipitate secondary IMHA.

Neoplastic disease is one such trigger, and associations between IMHA and lymphoma, myeloproliferative disease, anaplastic sarcoma, and hemangiosarcoma are well documented.^{30,37} The immunological mechanisms whereby neoplasia might trigger the production of RBC-reactive antibodies are not understood.

A trigger for IMHA of increasing importance is infectious disease. In particular, the canine arthropod-transmitted infectious diseases (babesiosis, ehrlichiosis, leishmaniosis, rickettsioses, and possibly anaplasmosis and bartonellosis) may all include IMHA as one part of their complex pathogenesis.^{6,26,50} These organisms establish a unique interaction with the host immune system that may result in a range of secondary immune-mediated phenomena not directly associated with the infection per se.⁵⁰ Although IMHA triggered by such

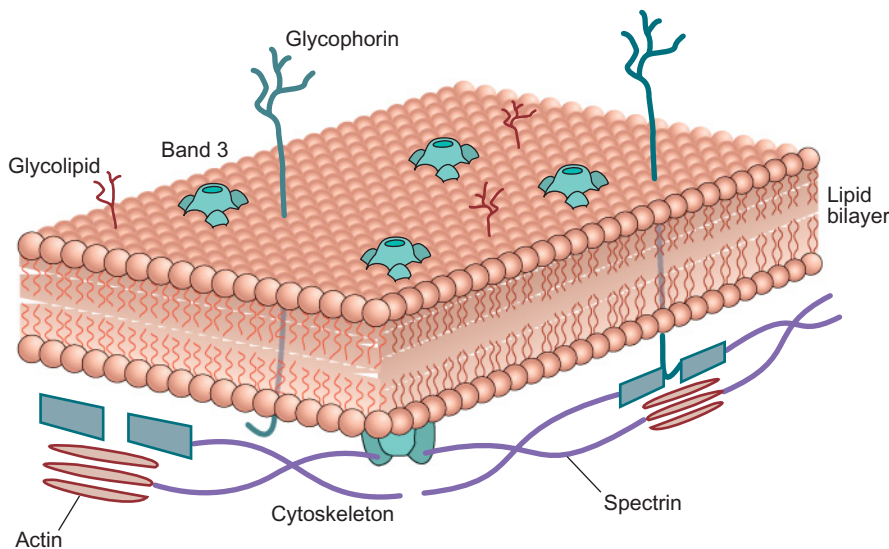


FIGURE 33.3 A schematic diagram of the RBC membrane showing the major target autoantigens in canine AIHA: the glycophorins, the anion exchange molecule (band 3) and the cytoskeletal molecule spectrin. There is conservation of some of these autoantigens across species. Band 3 may be recognized in dogs and humans with AIHA, and is the target of the autoimmune response in some experimental mouse models of the disease.

infection has been long recognized in traditionally endemic areas, recent increases in international pet animal movement, together with the background of climate change, means that these infectious diseases must now be considered in non-endemic geographical regions. The travel history of any dog presenting with anemia must be considered, and practitioners should remain updated on the progressive spread of these organisms and their vectors. There are now a number of documented instances of geographical extension of the canine arthropod-borne infections, for example the spread of leishmaniasis throughout the North American foxhound population,²⁰ and spread of babesiosis into northern European countries.^{5,34} Recent interest has been expressed in the potential for hemoplasmas to trigger IMHA in the dog.^{28,62} A single, small study of dogs with IMHA failed to detect bacteremia by blood culture as a potential trigger for the disease.⁴⁰

A further well-documented trigger for canine IMHA is drug therapy. Classically the potentiated sulphonamide drugs are incriminated in a wide range of breeds⁵⁵ and one experimental instance of cephalosporin-associated immune cytopenia has been documented.⁸ A single case study of carprofen-associated immune-mediated blood dyscrasia is reported.³⁸

The most controversial association is that proposed between the development of canine IMHA and vaccination in the immediately preceding 4-week period.¹⁷ Anecdotal evidence for this association has been available for some time;¹⁸ however, a pivotal study by Duval and Giger²¹ provided evidence for a temporal relationship of vaccine-associated IMHA in the dog. A large epidemiologic survey that was subsequently conducted in the United Kingdom failed to support these findings. In this study, there was no clear evidence that dogs with Coombs'-positive AIHA were more likely to have been recently vaccinated.¹

Overall, it appears that vaccine-associated IMHA does occur in dogs but with low incidence. The report of the United Kingdom Veterinary Products Committee provided specific data from a pharmacovigilance database which suggested that vaccine-associated IMHA was a relatively rare occurrence (incidence of 0.001 per 10,000 doses of vaccine sold).²⁴ The immunological mechanisms by which these reactions develop are not understood and no particular vaccine has been implicated.

Nonregenerative IMHA in the Dog

The immune response in dogs with IMHA may sometimes target the bone marrow erythroid precursors instead of, or in addition to, circulating RBCs. In this instance the bone marrow will fail to develop a regenerative response. Examination of the bone marrow becomes an essential component of the diagnostic work-up in animals with IMHA that do not have reticulocytosis after 5 days. A spectrum of such change is recognized and classified in different ways. Some reports distinguish the situation where there is impaired erythropoiesis but erythroid precursors are present

(NRIMHA), to that where there is virtual absence of erythropoiesis (acquired PRCA; see Chapter 38).⁵³ In NRIMHA the erythroid lineage may display either hyperplasia or maturation arrest at a specific point of the developmental lineage.²⁵

The diagnostic process in PRCA is similar to that described below, but affected dogs may be Coombs'-negative if the causative antibody that targets marrow precursors does not cross-react with antigens expressed by mature RBCs. In a retrospective study investigating nonregenerative IMHA in the dog, the Coombs' test was positive in 57%; 23% of these patients also tested positive for ANA.⁵³ There is no diagnostic test for the detection of precursor-associated antibody or complement, although the serum of such dogs has been shown to contain antibody that can inhibit erythropoiesis *in vitro*.⁵⁹

A range of bone marrow pathology has recently been described in dogs with PRCA, suggesting that the hemopoietic impairment involves changes in addition to antibody-mediated inhibition of erythropoiesis.⁶⁰ These reported changes include: dysmyelopoiesis, myelonecrosis, myelofibrosis, interstitial edema, hemorrhage, acute inflammation, hemophagocytic syndrome, lymphoid aggregation or plasma cell hyperplasia. These changes were more often present in the marrow of animals with NRIMHA with erythroid hyperplasia, than those with NRIMHA with maturation arrest, or in PRCA. The former subgroup of dogs also had reduced survival time compared with the two latter subgroups.

CLINICAL PRESENTATION

There are two main clinical presentations of canine IMHA. That most commonly recognized has a chronic onset (days to weeks) and is characterized by signs referable to anemia, erythrophagocytosis and immunological activity:

- weakness
- lethargy, exercise intolerance
- anorexia
- pyrexia
- pallor of mucous membranes
- tachypnoea, tachycardia
- hepatosplenomegaly
- lymphadenomegaly.

Acute onset (1–2 days) IMHA is less commonly recorded and is associated with:

- severe intravascular hemolysis with jaundice, hemoglobinemia and hemoglobinuria
- pyrexia
- vomiting.

A proportion of dogs with IMHA will subsequently develop disseminated intravascular coagulopathy (DIC) or dyspnea due to pulmonary thromboembolism.^{29,33} Thromboembolic disease may affect the spleen, kidneys, heart and lymph nodes in addition to the

lung.⁵⁸ Risk factors for development of these complications are reported as hyperbilirubinemia, a negative Coombs' test, elevated serum alkaline phosphatase, hypoalbuminaemia, cage confinement, recumbency and the presence of indwelling catheters.^{10,29,58} These complications arise following development of venous stasis, endothelial damage and a hypercoagulable state. It has been suggested that this effect may in part be associated with the presence of an anti-phospholipid antibody that enhances platelet aggregation and depresses regulation of the coagulation pathways.⁵² However, in a recent case series only two of 20 dogs had evidence of anti-phospholipid activity.⁴⁹

DIAGNOSIS

The diagnosis of IMHA proceeds through a series of laboratory tests after identification of compatible clinical history and presenting signs. An EDTA-anticoagulated blood sample should be collected for hematological examination. This should be examined for the phenomenon of autoagglutination that may be observed by rotating the collection tube, or by placing a drop of blood on a microscope slide. Autoagglutination may only occur at 4 °C, so blood should be refrigerated before making this assessment. True autoagglutination may be grossly distinguished from rouleaux formation by adding an equal volume of saline to the drop of blood. Rouleaux will be dispersed by this procedure but agglutination will persist. It is often said that a positive "in-saline slide autoagglutination test" provides definitive evidence for IMHA and precludes the need for a Coombs' test. However, as described below, the Coombs' test can provide additional valuable information and should always be requested where possible.

Standard hematological examination will demonstrate the features of anemia. Canine IMHA is often a severe disease with a packed cell volume (PCV) of less than 20%. The anemia is classically regenerative (see nonregenerative disease below) with reticulocytosis, polychromasia, anisocytosis, and nucleated RBCs as consistent features (Fig. 33.4). The absence of a regenerative response after 5 days suggests the possibility of bone marrow disease and warrants collection of a bone marrow aspirate and core biopsy. Microscopic evidence of autoagglutination may be found and the presence of significant numbers of spherocytes is strongly suggestive of immune-mediated RBC damage. IMHA is often accompanied by pronounced neutrophilia and left shift. The mechanism by which this arises is thought to involve tissue necrosis and the effect of pro-inflammatory cytokine (e.g. IL-1, IL-6 and TNF- α) production by activated macrophages on granulopoiesis. Platelet numbers will be adequate in primary AIHA, but significant reduction in platelet count may indicate concurrent IMT, thromboembolism or DIC. Dogs with IMHA may have a range of hemostatic abnormalities including prolonged prothrombin time and activated partial thromboplastin time, elevation of fibrin degradation products, elevation in D-dimer, and decrease in anti-thrombin

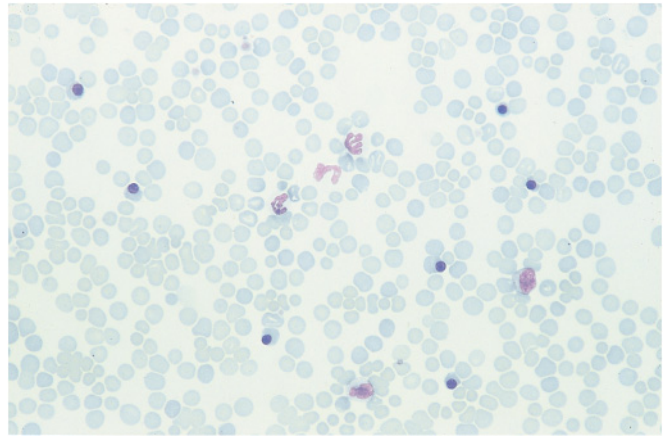


FIGURE 33.4 Blood smear from a dog with AIHA demonstrating the features of anisocytosis, polychromasia, spherocytosis and the presence of nucleated RBCs.

activity.^{10,49} Activation of circulating platelets as assessed by increased expression of membrane P-selectin is also reported.⁶¹

In the presence of clinical and hematological evidence for IMHA, the definitive diagnostic procedure is the Coombs' test (direct antiglobulin test, DAT) (Fig. 33.5). This test will demonstrate the presence of erythrocyte-bound immunoglobulin and/or complement, but does not distinguish between AIHA and secondary IMHA. The Coombs' test is performed with EDTA blood and there will be variation in the methodology used by different laboratories (see Chapter 140). A full Coombs' test will be performed using a polyvalent Coombs' reagent (that recognizes IgG, IgM and complement C3), but also with antisera specific for each of these immunoreactants alone. The read-out for the test is erythrocyte agglutination, and the titer of each positive reaction should be determined. The incidence of false negative reactions is greatly reduced when the full test is performed in this manner. Recent studies have shown that when the test is only performed using polyvalent reagent at 37 °C a proportion of dogs with IMHA will be falsely Coombs' negative.^{43,57}

In general terms, two broad patterns of Coombs' reactivity are identified and these have some correlation with clinical presentation.⁵⁷ The most commonly recorded pattern involves an IgG antibody that may be present with IgM and/or C3, and that reacts equally at 4 °C and 37 °C. This pattern frequently correlates with disease of chronic onset and is compatible with extravascular RBC removal. Occasionally, a cold-reactive IgM antibody is identified that may fix complement and occurs in the absence of IgG. Such cold-agglutinating IgM antibodies generally elute from the surface of the RBCs at physiological temperature. The clinical significance of these antibodies is often debated, but this pattern of Coombs' reactivity is more often associated with sample autoagglutination, intravascular hemolysis and acute onset, severe clinical disease. A recent study also suggests that cold-reactive IgM antibodies may more frequently associate with secondary IMHA.⁵⁷

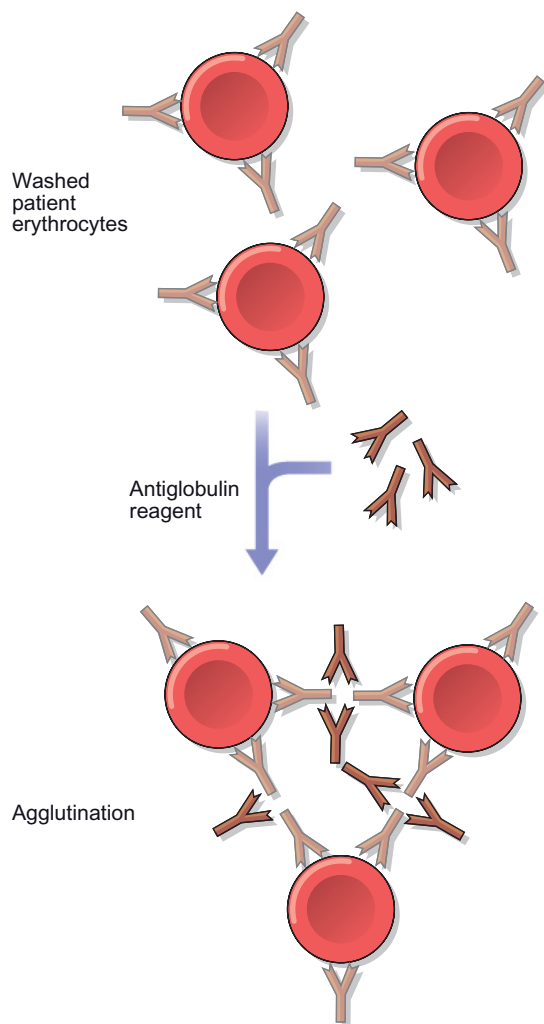


FIGURE 33.5 Principle of the Coombs' test. A suspension of washed patient RBCs with membrane immunoglobulin or complement attached *in vivo*, is incubated with an antiglobulin reagent that cross-links these immunoreactants, forming a lattice that appears grossly as an agglutinate of RBCs.

Such cold agglutinins may be pathogenic, rather than simply an *in vitro* phenomenon, if their thermal reactivity extends to the sub-physiological range. The clear example of clinical relevance of these antibodies is in the relatively rare "cold agglutinin disease", where IgM hemagglutinins result in the formation of microthrombi that occlude peripheral capillaries in the skin of the ear and tail tip. Predisposed patients will therefore develop necrosis of these sites when inadvertently exposed to freezing temperatures. There is no clear association between the titer of RBC-bound antibody and disease severity. Although low-titers or the presence of complement alone on the RBC surface are often thought to be more consistent with secondary IMHA than AIHA, a recent study has not confirmed this.⁵⁷

The Coombs' test remains the "gold standard" for diagnosis of IMHA; however, other tests are reported. The indirect Coombs' test (for detection of circulating

autoantibody by incubation with normal erythrocytes) is not considered valid for use in veterinary species due to the low prevalence of non cell-bound autoantibody.⁵⁶ A cell-ELISA based method (DELAT) has been described, but is a time consuming procedure and is largely used as a research tool.³ Recent studies have demonstrated the utility of flow cytometry for detection of RBC-bound antibody. Flow cytometric testing has good sensitivity and specificity when compared to the Coombs' test, but is limited by availability of this equipment.^{45,63}

A recent innovation is the development of a tube-based test designed for in-practice use.⁵⁶ This rapid assay involves the use of polyvalent reagent and gives a simple positive or negative outcome, and validation studies show good agreement with the more definitive Coombs' test (MJ Day, unpublished data).

Other immunodiagnostic procedures may be positive in dogs with IMHA. Some cases are reported to have significant serum titers of ANAs. In the series presented by Weinkle et al.⁵⁸ 39% of dogs with IMHA had a serum ANA titer >80. In the presence of concurrent thrombocytopenia, tests for platelet autoantibody should be requested, although this is not routinely possible in many areas of the world. Adjunct immunodiagnostic tests such as determination of serum complement concentration (decreased levels of C3 or C4 may reflect deposition on RBC membranes) or serum IgA concentration (IgA deficiency may be associated with canine autoimmunity) may be requested.^{54,56}

THERAPY

Management of canine IMHA is dependent on whether the disease is primary or secondary in nature, the latter necessitating appropriate therapy for any underlying infectious or neoplastic trigger. The first line approach to therapy for canine idiopathic IMHA involves the use of tapered immunosuppressive doses of glucocorticoids (e.g. prednisolone commencing at 2–4 mg/kg daily). In dogs with severe, acute-onset intravascular hemolysis, or with nonregenerative IMHA, or with anemia that is non-responsive to glucocorticoid, the addition of cytotoxic agents (e.g. cyclophosphamide 50 mg/m² orally for the first 4 days of each week for 4–5 months only; azathioprine commencing at 2 mg/kg daily) to the regime is indicated. Recent studies have questioned the efficacy of adjunctive cyclophosphamide in therapy for AIHA and suggested that in fact this combination may worsen disease outcome. For this reason cyclophosphamide adjunct therapy has now largely fallen out of favour.^{9,27,35} Where a standard combination immunosuppressive protocol of prednisolone and azathioprine was used in one large case series, effective control was reported with 3 months duration of treatment in the majority of dogs.⁴⁴

In severely anemic dogs (PCV < 10%), supportive therapy in the form of cross-matched whole blood or packed RBC transfusion may be required. The benefits

of transfusion in providing short-term oxygen carrying capacity outweigh any possibility of enhancing hemolysis by providing greater antigenic load. The use of polymerized bovine hemoglobin has also been investigated but some studies suggest this may contribute to poor clinical outcome²⁷ although this finding is not corroborated by others.⁵⁸ A number of other adjuncts to glucocorticotherapy are documented. The use of danazol (5 mg/kg orally twice daily) is controversial as recent studies have suggested that this drug may have little beneficial effect.^{27,39} Similarly, combination therapy with glucocorticoid and ciclosporin (5 mg/kg daily) appears to provide no greater benefit than glucocorticoid monotherapy.²⁷ The use of intravenous human gammaglobulin (0.5–1.5 g/kg given over 4 hours), to block macrophage Fc receptors or bind circulating autoantibody, is of documented benefit, but an expensive undertaking.⁴⁸ There is no consistent evidence that splenectomy (to remove a major site of extravascular hemolysis and autoantibody production) has any beneficial effect, although occasional early reports proposed this procedure.²³ In dogs with acute, severe AIHA the use of prophylactic heparin or ultra-low dose aspirin treatment to prevent the complications of DIC or pulmonary thromboembolism is advocated. A number of such prophylactic regimes are described.⁵⁸

OUTCOME

Recent studies have evaluated clinical outcome in canine IMHA.^{44,46,58} It is clear that canine IMHA must be regarded as a severe disease, as in the referral setting, around 50% of animals will die during initial hospitalization. Death is more likely to occur in patients with secondary or acute onset disease.^{41,57} Factors associated with reduced survival in the large case series of Weinkle et al.⁵⁸ included:

- autoagglutination
- high serum bilirubin ($>15.0 \mu\text{mol/L}$)
- left shift neutrophilia (bands $\geq 3.0 \times 10^9/\text{L}$)
- thrombocytopenia (platelets $<150 \times 10^9/\text{L}$)
- hypoalbuminaemia (serum albumin $<30.0 \text{g/L}$)
- hypokalemia (serum potassium $<3.5 \text{mmol/L}$)
- elevated serum creatine kinase ($>250 \text{U/L}$).

By contrast, some dogs with IMHA may make excellent recovery with appropriate supportive and immunosuppressive therapy, but remain at risk for disease relapse. There is often very rapid clinical and hematological response to therapy in such patients; however, serial monitoring of the Coombs' test has revealed striking persistence (for many months) of RBC-bound autoantibody in many cases (Fig. 33.6).¹⁵ Relapses may occur months or years after the initial episode, and are often more severe, resulting in death. The median survival time in most studies is just over 1 year, but in one large case series, approximately 25% of patients had excellent long-term survival.⁵⁸ Other manifestations of autoimmunity may surface in dogs recovered from

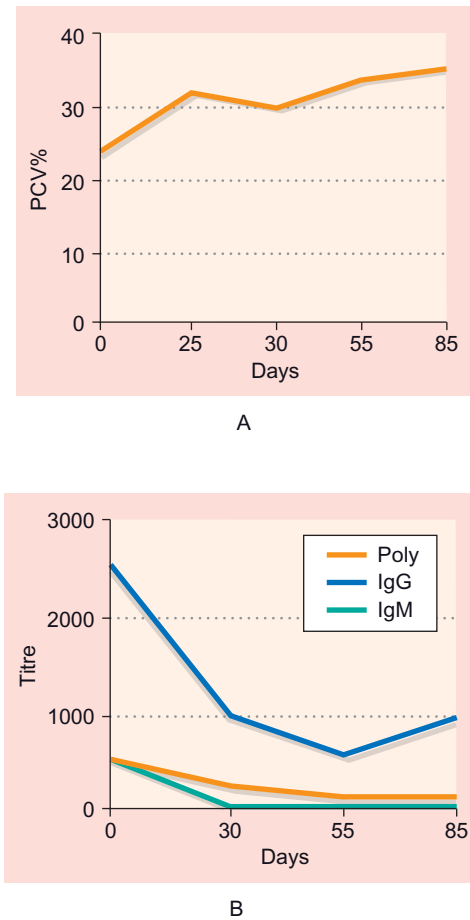


FIGURE 33.6 Serial monitoring of IMHA in a dog with IMHA. The dog was treated with prednisolone at an initial dose of 2 mg/kg daily that was gradually tapered and withdrawn on day 76. Despite the rise and plateau in packed cell volume (A), the dog remained Coombs'-positive throughout the period of monitoring (B). In B, serial titres obtained using polyvalent canine Coombs' reagent, anti-dog IgG and anti-dog IgM are shown. (Adapted from Day MJ. Serial monitoring of clinical, haematological and immunological parameters in canine autoimmune haemolytic anaemia. *J Small Anim Pract* 1996;37:523–534, with permission).

IMHA, sometimes several years later. For example, dogs may present with IMT (and no anemia) subsequent to IMHA (without thrombocytopenia). SLE and immune-mediated skin disease are also reported sequels.

Therefore, recovery from IMHA necessitates regular monitoring for the life of the patient. Hematological monitoring is the most cost-effective means; it is recommended that a PCV be performed every 2 weeks throughout the course of treatment, extending for 6–12 months after withdrawal of therapy. In dogs recovered from IMHA associated with vaccination, care should be taken with administration of subsequent booster vaccines. A risk-benefit analysis should be conducted in consultation with the client. Measurement of serum antibody titre to core virus components may be performed to support any decision not to re-vaccinate, but

where re-vaccination is required, use of a product from a different manufacturer and a product with the longest possible duration of immunity is advised. There is, however, little clear evidence that booster vaccination induces relapse of disease or disease of greater severity; such studies remain to be performed.

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Immune-Mediated Anemias in the Cat

TRACY STOKOL

Pathogenesis
Clinical Signs
Diagnosis
Treatment
Primary IMHA

Secondary IMHA
Infectious Agents
Neoplasia
Drugs
Miscellaneous
Cold Agglutinin Disease
RBC Blood Group Incompatibilities

Acronyms and Abbreviations

C3, complement component 3; Fc, fragment crystallizable region of the immunoglobulin heavy chain FeLV, feline leukemia virus; Hct, hematocrit; IFN- γ , interferon- γ ; IgG, immunoglobulin G; IL-10, interleukin 10; IgM, immunoglobulin M; IMHA, immune-mediated hemolytic anemia; MHC, major histocompatibility; PRCA, pure red cell aplasia; PTU, propylthiouracil; RBC, red blood cell; T, T helper cell; Tr, regulatory T helper cell.

Immune-mediated hemolytic anemia (IMHA) is an uncommon hematological disorder in cats. IMHA is usually caused by the binding of antibodies to mature red blood cell (RBC) membranes, causing their accelerated and premature removal from the circulation by mononuclear phagocytes. In some cases, the immune attack is thought to be directed against erythroid progenitors in the marrow, resulting in a nonregenerative anemia from ineffective erythropoiesis or pure red cell aplasia (PRCA).^{46,51} If the initiating cause of IMHA is unknown, the disease is called primary or idiopathic (this condition has also been termed autoimmune hemolytic anemia; see Chapter 33). When IMHA is initiated by an underlying disease (e.g. infectious agents, neoplasia) or drugs, it is called secondary IMHA. Hemolytic transfusion reactions and neonatal isoerythrolysis are also immune-mediated conditions, caused by blood group incompatibilities.

Pathogenesis

IMHA is considered a type II immune complex hypersensitivity reaction, in which there is a failure of self-tolerance.⁵ Antibodies, usually IgM or IgG, react with antigens in the RBC membrane. Once the antibodies bind to RBCs in the circulation, they can activate complement. RBCs are then destroyed through Fc- and complement-receptor-mediated phagocytosis by macrophages in the spleen (primarily), liver and bone

marrow (i.e. extravascular hemolysis) and/or through direct complement-mediated lysis (i.e. intravascular hemolysis) (Fig. 34.1). In cats, hemolysis is primarily extravascular, with many cats displaying autoagglutination of RBCs.^{32,44,56} Intravascular hemolysis, with hemoglobinemia and hemoglobinuria, is rare and usually secondary to exposure to incompatible RBC blood types.^{4,24}

Substantial progress has been made in the understanding of the basic pathogenesis of classic IgG-mediated IMHA from studies in humans and genetically-modified mice. Antibody production in IgG-mediated IMHA is a T helper (specifically Th1) cell-dependent phenomenon. It is now thought that IMHA results from the proliferation of auto-reactive Th1 cells (perhaps through unmasking of a cryptic antigen or more efficient processing and presentation of RBC antigens by dendritic cells) combined with suppression of T regulatory cells (Tr), which normally inhibit auto-reactive Th1 cells (Fig. 34.1).⁵ The underlying mechanisms responsible for altered antigen processing or unmasking of cryptic antigens are unknown. Antigens that induce IMHA can be intrinsic to the RBC ("self" antigens) or can be foreign proteins (e.g. drugs or infectious agents) that bind to or modify RBCs, rendering them antigenic, or that potentially express similar antigenic determinants to RBCs. Specific RBC antigens responsible for IMHA in cats have not been identified.

Expression of a normally “cryptic” RBC antigen on the surface of dendritic cell stimulates auto-reactive T helper cell (Th)

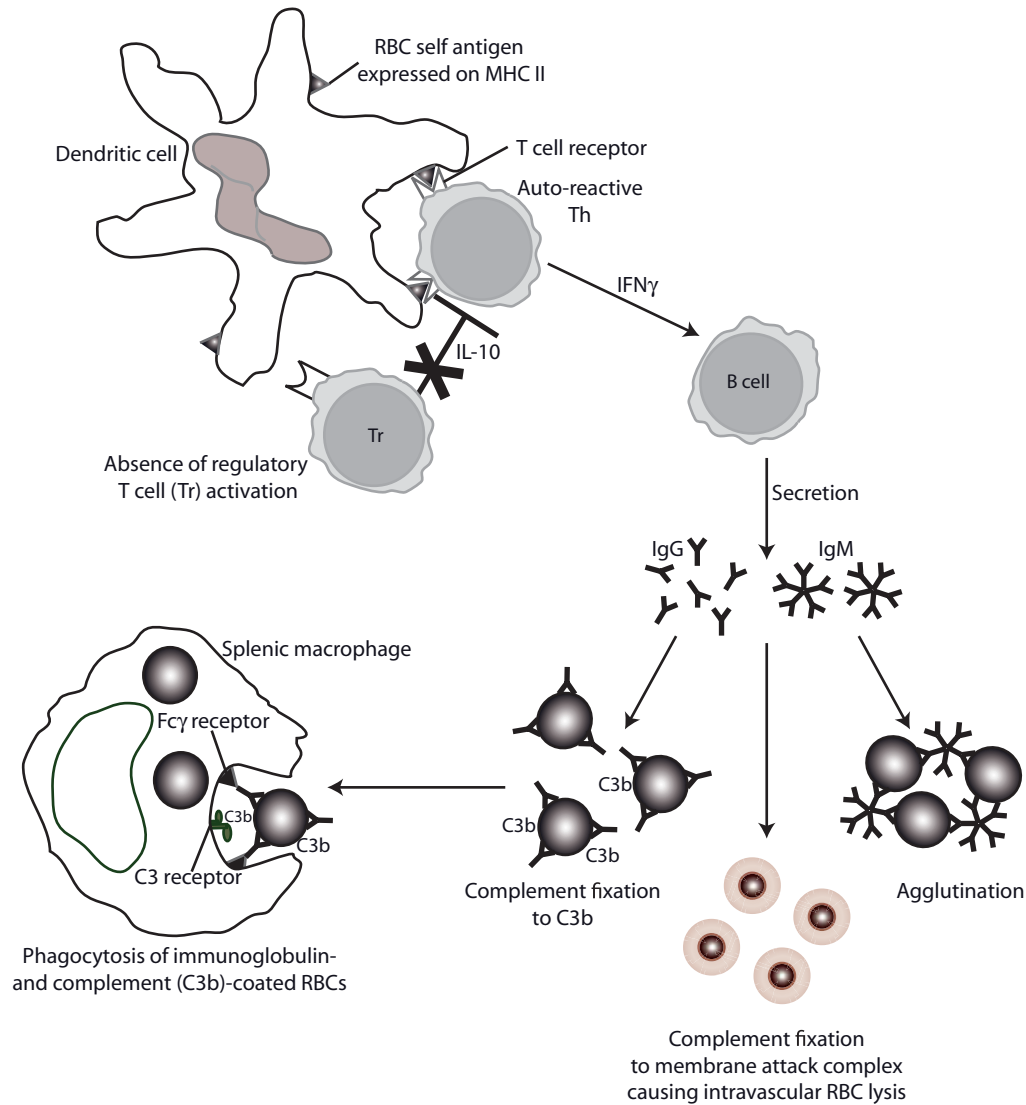


FIGURE 34.1 A self-RBC antigen (which is normally inefficiently or not processed) is expressed on the surface of a dendritic cell or other antigen-presenting cells in conjunction with major histocompatibility type II (MHC II) proteins. The self-RBC antigen and MHC II (not shown) are recognized by T cells and co-stimulatory receptors (not shown) on auto-reactive T helper cells. Concurrently, regulatory T cells (which normally keep the immune system in check through secretion of interleukin-10 [IL-10]) are suppressed, leading to uncontrolled proliferation of auto-reactive T helper cells. These T helper cells activate B cells, via a Th1-driven response (through secretion of IFN γ). B cells secrete immunoglobulins (IgM and/or IgG) that specifically recognize the RBC self-antigen. Activation of complement (by IgM or certain classes of IgG), through the classical pathway, leads to the binding of C3b on RBCs. Macrophages in the spleen, liver and bone marrow recognize the Fc portion of immunoglobulin and bound C3b through specific receptors and remove the immunoglobulin- and C3b-coated RBCs prematurely from the circulation. Efficient complement-fixing antibodies can result in the formation of the membrane attack complex of complement (C6-9) on RBCs, causing them to lyse intravascularly. Alternative mechanisms by which Th1 cells can be activated are processing of a foreign peptide that cross-reacts with RBC antigens or microbial superantigens that cause hyper-stimulation of the immune system.

T cell dysregulation also likely underlies nonregenerative variants of IMHA, including PRCA,²¹ although there is no definitive proof for this in cats. In nonregenerative IMHA, the inciting RBC antigen is expressed exclusively or concurrently on RBC progenitor cells,^{12,22} resulting in their intramedullary destruction. Also, antibodies that target erythropoietin can result in PRCA.^{18,21} Although the focus on IMHA pathogenesis has been on

antibody-mediated destruction of RBCs, cell-mediated immune mechanisms may also be operative, particularly in nonregenerative forms of IMHA, such as PRCA.²¹ Cytotoxic T and natural killer cells (granular lymphocytes) can directly kill (in an antibody-dependent or -independent fashion) RBC progenitor cells through direct lysis or apoptosis. Indeed, cell-mediated cytotoxicity is thought to be responsible for some cases

of acquired PRCA in humans associated with neoplastic or non-neoplastic expansions of these lymphocytes.²¹ Alternatively, activated T cells or macrophages can suppress rubriblast proliferation through secretion of inhibitory cytokines, such as interferon- γ (IFN γ) and tumor necrosis factor- α .^{17,21}

Clinical Signs

Clinical signs in cats with primary and secondary IMHA are primarily attributable to the anemia and consist mostly of lethargy and inappetence. Some cats also suffer from vomiting, pica, constipation or obstipation, and polydipsia.^{32,44,46,56} On physical examination, cats have mucous membrane pallor and are usually afebrile, with heart murmurs secondary to anemia. Some cats have mild organomegaly (mostly affecting the spleen) or icterus.^{32,44} Cats with secondary IMHA may display additional clinical signs related to the underlying primary disease.

Diagnosis

Because clinical signs and physical examination findings are not specific, the diagnosis of IMHA (primary or secondary) relies on confirming an immune-mediated pathogenesis for the anemia. This is accomplished by identifying autoagglutination, documenting positive immunologic testing (specifically a positive direct Coombs' test), or a favorable response to immunosuppressive therapy. Spherocytes, which are sensitive indicators of IMHA in dogs,^{16,49} are difficult to identify in cats, due to their small RBCs which normally lack central pallor.

Autoagglutination of whole blood is seen in many cats with IMHA.^{32,44,51,56} However, RBC rouleaux formation (secondary to increased fibrinogen and/or immunoglobulins) can mimic agglutination and it is important to confirm grossly visible agglutination on diluted whole blood or washed RBCs. Typically a 1:4 dilution of whole blood with isotonic or phosphate-buffered saline or washing of RBCs should disperse rouleaux, whereas agglutination persists.⁵⁶ However, weak avidity antibodies may be eluted from RBCs during dilution, resulting in a false negative agglutination reaction. Agglutination should ideally be confirmed by microscopic examination of the sample, where agglutinated clumps of RBCs can usually be distinguished from rouleaux. Agglutination is typically considered *prima facie* evidence of IMHA; however, it can rarely be observed as an artifact secondary to EDTA-associated binding of a non-pathogenic antibody to RBCs.⁴²

Because most pathogenic antibodies are warm-reacting IgG, a direct Coombs' test (performed at 37°C) is frequently positive in cats with primary or secondary IMHA.^{13,32,35,40,44,51,56} However, if low numbers or weak avidity antibodies are attached to RBCs or antibodies only recognize epitopes expressed on RBC progenitor cells, the direct Coombs' test may be negative, as reported for up to 50% of cats with nonregenerative

IMHA.^{2,46} Direct Coombs' testing at 4°C is not recommended, since RBCs from healthy cats can yield a positive reaction due to the existence of non-pathogenic cold-reactive antibodies.^{20,29}

In the absence of autoagglutination or a positive direct Coombs' test, the diagnosis of IMHA depends on a positive response to immunosuppressive therapy. This approach has been used to diagnose cases of primary and secondary IMHA in cats.^{33,46,57}

Treatment

Primary IMHA is treated with immunosuppressive drugs, typically corticosteroids. Other immunosuppressive agents (e.g. cyclosporin, intravenous IgG) may be added if the anemia remains non-responsive. Adjunctive supportive therapy, including blood transfusions and gastroprotective drugs, is often required. Most cats develop a regenerative response (increase in aggregate reticulocytes or increases in hematocrit [Hct]) within 1 month of treatment. Relapses can occur when immunosuppressive therapy is tapered, particularly if done abruptly or rapidly. The prognosis with primary IMHA is good, with >60% of cats surviving for several months to years after diagnosis. The regeneration status does not appear to influence the response to treatment.^{2,31,32,46,57} Cats with secondary IMHA should be treated for the underlying disease. This alone (particularly in the case of drug-induced IMHA) may result in resolution of the hemolytic process and the anemia; however, additional corticosteroid therapy may be required.^{3,8,18,40,41,44}

PRIMARY IMHA

Primary IMHA is defined as an immune-mediated anemia of unknown cause; therefore, it is a diagnosis of exclusion. It is an uncommon disease in cats, with only three published retrospective case series of 9–57 affected cats in a 6–10 year period,^{32,46,51} and two abstracts of 25–36 affected cats over a 10–18 year period.^{2,31} In three of these studies,^{31,32,51} the diagnosis of IMHA was made on the basis of autoagglutination and/or a positive direct Coombs' test without evidence of underlying disease in anemic cats. In one study on PRCA, the diagnosis was primarily based on response to immunosuppressive therapy.⁴⁶ Two other retrospective studies of 7 and 21 cats consisted mostly of cats with secondary IMHA, with only 14–28% likely having primary IMHA.^{44,56} The diagnosis in these latter two studies was accomplished by direct Coombs' testing or autoagglutination. Although the occurrence of primary IMHA may be quite low as indicated by the paucity of reports, the reliance on a positive direct Coombs' test or autoagglutination for diagnosis may cause the true disease incidence to be underestimated. Since these tests are not 100% sensitive for IMHA in other species,⁴⁹ it is unlikely that all cases of feline primary IMHA will have positive results, particularly the nonregenerative variants.

Primary IMHA is typically considered to result in a regenerative hemolytic anemia (with concurrent

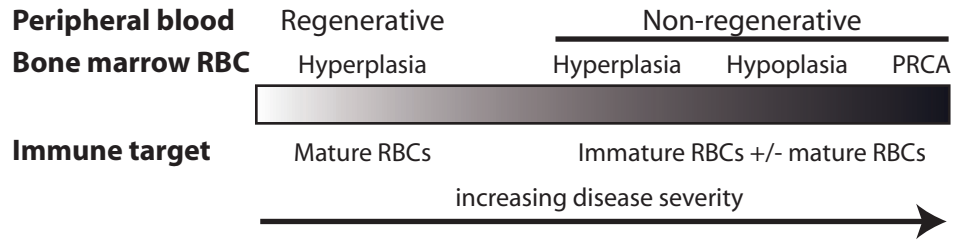


FIGURE 34.2 IMHA forms a continuous spectrum of disease, ranging from the classical presentation of a regenerative anemia with erythroid hyperplasia in the bone marrow to the most severe clinical manifestation, a nonregenerative anemia due to the lack of RBC progenitors in the bone marrow (i.e. pure red cell aplasia). IMHA can also present between these two extremes as either a weakly regenerative anemia or a nonregenerative anemia associated with bone marrow erythroid hyperplasia or hypoplasia.

erythroid hyperplasia in the bone marrow); however, the anemia may be nonregenerative.^{2,32,36,46,51,57} In one study, the anemia was nonregenerative in 58% of 19 cats with primary IMHA.³² Lack of regeneration may be due to duration, disease severity, presence of concomitant disease, or antigen specificity (Fig. 34.2). If the bone marrow has had sufficient time to respond (i.e. the anemia has been present for >5 days), the absence of regeneration has been attributed to ineffective erythropoiesis or PRCA (see below).^{46,51} These two bone marrow conditions appear to occur with similar frequency in cats with nonregenerative IMHA.^{2,51} Since bone marrow aspirates were not done in the 19 cats of one report³² and some cats had been sick for <5 days, the cause of the nonregenerative nature of the anemia in these cats is uncertain.

Regardless of the form of primary IMHA (regenerative or nonregenerative), the disorder occurs mostly in young (<3 years), domestic shorthair cats of both sexes. The anemia is usually normocytic, normochromic, and severe (Hct < 20%).^{2,32,36,46,51,57} Leukocyte and differential cell counts are usually within reference limits, although some cats can have a concurrent neutropenia or lymphocytosis.^{2,31,32,51} Thrombocytopenia has been identified in several cats.^{2,31,32,51,57} Thrombocytopenia and neutropenia are more frequently seen in cats affected with ineffective erythropoiesis than those with PRCA.^{46,51} Some cats (30% of 10 tested cats) in one study had prolonged screening coagulation times and were suspected or clinically diagnosed with disseminated intravascular coagulopathy;³² however, clinical thrombosis is rarely recognized.³⁸ Cats can have a mild hyperbilirubinemia (supporting an extravascular hemolytic process),^{31,32} although this is uncommon in PRCA.⁴⁶ There is also evidence of hypoxic liver injury in about half of the cats.^{32,46} Hyperferremia with normal total iron binding capacity is a consistent finding in cats with PRCA.⁴⁶

In cats with nonregenerative anemia, bone marrow aspirates are required to distinguish between ineffective erythropoiesis and PRCA and to help exclude other causes, including primary or infiltrative bone marrow neoplasia, reactive histiocytic disorders, and myelodysplastic syndromes. In one study, 60-day survival rates were lower in cats with ineffective erythropoiesis compared to PRCA (although treatment details were not provided),⁵¹ highlighting the importance of bone marrow aspirates in these cats. Ineffective erythropoi-

esis is characterized by erythroid hyperplasia in a typically hyperplastic marrow. Erythroid maturation may be left-shifted (with more immature than mature precursor cells) or incomplete. Incomplete maturation has also been called “maturation arrest”, a term which should be avoided, because there is no evidence that the absence of late stage erythroid precursors is due to a true arrest in maturation. There is minimal evidence of dysplasia in cats with incomplete maturation of erythroid precursors.⁵¹ PRCA is defined as <5% erythroid progenitor cells in the bone marrow. A consistent bone marrow finding in cats with ineffective erythropoiesis and PRCA (86–100% of cats) is a lymphocytosis of small lymphocytes (15–55% of total cells), found diffusely or in aggregates.^{46,51} Based on one study, these lymphocytes appear to be mostly B cells (identified by CD19 immunostaining).⁵² However, this marker has not been validated in cats³⁷ and only four cats were evaluated in this study; therefore these results should be verified by immunophenotyping additional cases with accepted feline B cell markers.³⁷ Such bone marrow lymphoid aggregates are also not specific for primary IMHA, because they can be seen in cats with various infectious and non-infectious diseases.^{9,15,52} Unlike dogs,⁴⁷ myelofibrosis is quite rare. In one study, only 4% of 57 cats with IMHA had evidence of collagen fibrosis.⁵¹ Ineffective erythropoiesis and PRCA likely represent extreme variations on a common theme, rather than separate entities. Indeed, animals whose bone marrow findings are not consistent with the above two categories (e.g. those with erythroid hypoplasia) likely fall between these two extremes (Fig. 34.2).

The main differential diagnoses for ineffective erythropoiesis are secondary IMHA (induced by drugs, infectious disease, or underlying neoplasia) and myelodysplastic syndromes (also called primary myelodysplasia), a clonal disorder of hematopoiesis (see Chapter 64).^{7,28} Secondary IMHA can be distinguished by testing for underlying disorders (including PCR-based tests for hemotropic mycoplasma) and historical details. Cats with myelodysplastic syndromes typically have prominent dysplastic features in one or more hematopoietic cell lines, more frequent myelofibrosis, and iron is often evident in mature (siderocytes) and immature (sideroblasts) erythroid cells.^{6,7} Individual cases of ineffective erythropoiesis due to nonregenerative IMHA may have mild dysplastic features in hematopoietic cells⁵¹ which

can make distinction difficult. In these cases, confirmation of a diagnosis of nonregenerative IMHA may depend on a favorable response to immunosuppressive therapy and negative test results for feline leukemia virus (FeLV), which may be positive in myelodysplastic syndromes.⁷ PRCA can also occur secondary to drugs, specifically recombinant erythropoietin,^{18,41} and FeLV infection.^{34,45} These causes can potentially be distinguished by historical details and FeLV testing. Furthermore, FeLV-induced PRCA is mostly an experimental disease (see below).⁴⁵

SECONDARY IMHA

The diagnosis of secondary IMHA is based on the same criteria as primary IMHA (i.e. positive Coombs' test, autoagglutination, and favorable response to immunosuppressive therapy); however, an underlying disease is identified in affected cats or they have been given an incriminating drug.^{32,40,41,44,56} The most frequent diseases associated with secondary IMHA are infectious agents (e.g. FeLV and *Mycoplasma hemofelis*) and neoplasia.^{32,44,56} Animals with secondary IMHA often respond to immunosuppressive therapy, along with treatment directed at the primary disease or drug withdrawal. It should be noted that the presence of IgG bound to RBCs or a positive Coombs' test (particularly if weakly positive) does not always mean that the pathogenesis of an anemia is immune-mediated. For example, cats with anticoagulant rodenticide toxicity and inflammatory disease (e.g. cholangiohepatitis, pancreatitis) can have a positive direct Coombs' test.^{20,32} RBCs from cats with induced sterile abscesses show enhanced binding of IgG (particularly on older RBC fractions), which may be secondary to neutrophil-induced RBC oxidant injury.^{53,55} Although IgG-mediated extravascular hemolysis may decrease RBC survival and contribute to the early mild decrease in Hct (average 8% decrease within 3–7 days of abscess induction) in these cats,⁵⁴ immunosuppressive therapy is not warranted.

Infectious Agents

FeLV infection was the most common underlying disease in two retrospective studies of cats with IMHA, being identified in 42–71% of 7 and 21 cats, respectively.^{44,56} A regenerative anemia occurs in cats experimentally infected with FeLV subgroups A and B.³⁴ A positive direct Coombs' test (at 37°C) was identified in 83% of 12 anemic FeLV-positive cats in another study.²⁰ However, in most affected cats the anemia was attributed to primary myelodysplasia rather than IMHA. It is likely that the anemia in some FeLV-infected cats is immune-mediated, because the anemia can resolve with immunosuppressive therapy.⁴⁴ Experimental infection with FeLV subgroup C virus can induce PRCA in cats;³⁴ however, this does not appear to be immune-mediated and has been attributed to inhibition of early erythroid progenitor cells.¹ FeLV subgroup C is rarely isolated from naturally FeLV-infected cats with PRCA.⁴⁵

In fact, all reported cases of immune-mediated PRCA in cats were FeLV negative,^{36,46,51,57} indicating that FeLV is likely not involved in the pathogenesis of the majority of feline PRCA cases.

IMHA can occur in cats experimentally or naturally infected with *Mycoplasma hemofelis*,^{35,56,58} and corticosteroids are common adjunctive therapy in infected cats.²⁷ A nonregenerative anemia that responded to doxycycline and corticosteroid therapy occurred in two cats with *Ehrlichia* infections.⁸ A positive RBC agglutination test has been documented in 16% of cats naturally infected with *Babesia felis*.⁴³ Taken together, these findings suggest that an immune-mediated component may contribute to the anemia in cats with various infectious diseases.

Neoplasia

Hematopoietic neoplasia (lymphoma, myelodysplastic syndromes, and acute myeloid leukemia) has been associated with positive direct Coombs' test results or secondary IMHA.^{20,32,44,56} However, because most affected cats in these reports had concurrent FeLV infection, it is unclear if the neoplasia was directly responsible for the secondary IMHA. In humans, PRCA can be a paraneoplastic response associated with lymphoid and other solid neoplasms (e.g. thymoma).²¹ However, this has not been reported in the cat.

Drugs

Various mechanisms have been proposed as causes of drug-induced IMHA (Table 34.1).^{19,39} Although any drug has the potential to cause secondary IMHA (regenerative or nonregenerative), such reactions have been reported infrequently in cats. Drug-induced IMHA has been identified in cats treated primarily with propylthiouracil (PTU) for hyperthyroidism and recombinant erythropoietin for anemia of renal failure (see Chapter

TABLE 34.1 Mechanisms of Drug-Induced Secondary Immune-mediated Hemolytic Anemia

Hapten	Antibodies primarily recognize the drug, which is bound to RBC proteins. The RBC is subsequently destroyed as an innocent bystander
Immune complex	Antibodies (usually IgM) primarily recognize the drug-RBC protein complex
Autoimmune	Anti-RBC or anti-erythropoietin antibodies are induced by the drug (due to probable cross-reactivity). Hemolysis can proceed in the absence of the inciting drug
Adsorption	Drugs can induce the non-specific binding of immunoglobulins, complement components and other proteins to RBC membranes. Although this can result in a positive direct Coombs' test, RBC survival is usually unaffected

16).^{3,18,40,41,48} A severe anemia and thrombocytopenia developed in 9% of 105 hyperthyroid cats administered PTU. In all seven tested cats, the direct Coombs' test was positive. The anemia resolved with drug withdrawal and corticosteroid therapy.⁴⁰ PTU induced a regenerative hemolytic anemia, which was direct Coombs' test positive, within 3–8 weeks after initiation of PTU therapy in 40% of healthy cats. The anemia resolved and the Coombs' test became negative within 1–4 weeks after drug withdrawal and did not recur when a non-sulfated derivative of the drug was administered.³ PRCA develops in some cats treated with recombinant erythropoietin, of both human and feline origin, presumably due to anti-erythropoietin antibody production.^{18,41,48} This unexpected reaction with recombinant feline erythropoietin may be due to allelic and amino acid differences in natural and recombinant feline erythropoietin.⁴¹

Miscellaneous

Other immune-mediated conditions that have been associated with secondary IMHA include presumptive graft versus host disease in a cat after bone marrow transplantation¹³ and systemic lupus erythematosus.³³

COLD AGGLUTININ DISEASE

Cold agglutinin disease is really a hemolytic anemia induced by antibodies whose thermal reactivity is below 37°C. In human patients, the antibody is usually a monoclonal IgM, produced in association with non-Hodgkin's lymphoma. The antibody binds to RBC in the extremities (28–31°C) and fixes complement. Although this may result in mild intravascular hemolysis, usually the IgM elutes off RBCs at warmer core temperatures. However, the fixed complement (usually C3b) remains attached causing a chronic extravascular hemolytic anemia. In some cases, the monoclonal IgM may agglutinate RBCs in vessels in the extremities, causing ischemic necrosis. Cold hemolytic anemia can also occur as a transient disease with viral and bacterial (*Mycoplasma pneumoniae*) infections in people. In these cases, the antibody is usually polyclonal.^{19,23,39}

Cold hemolytic anemia is poorly recognized in the cat. IgM cold agglutinins have been identified in cats with experimental *Mycoplasma hemofelis* infections and were suspected to contribute to the anemia in these cats.⁵⁸ Cold agglutination was suspected (on the basis of a positive slide agglutination reaction in blood diluted 1:1 in saline) in a cat with tail and ear tip necrosis. However, the clinical signs in the cat resolved with removal of a lead foreign body.²⁵ Tail tip necrosis in kittens with suspected neonatal isoerythrolysis was also attributed to cold agglutination.¹⁰ Autoagglutination or a low titer direct Coombs' test that is only positive at 4°C should be interpreted with caution, since both humans and cats can have non-pathogenic cold-reactive antibodies that can cause these reactions.^{19,20,23,29,39}

RBC BLOOD GROUP INCOMPATIBILITIES

Three main blood types are present in cats: A, B and AB (see Chapter 92).^{11,26} Recently, a fourth blood type, *Mik*, was identified.⁵⁰ Immune-mediated destruction of RBCs through intra- and extravascular hemolysis occurs in cats exposed to incompatible blood types through transfusions or in utero during pregnancy (neonatal isoerythrolysis).^{4,11,14,24,30} This hemolytic reaction does not require prior sensitization, because cats contain naturally occurring antibodies against RBC antigens.^{11,24,50} These reactions are most severe in type B cats exposed to type A or AB blood, due the presence of strong agglutinins and hemolysins in these cats (see Chapter 100).^{11,24}

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Immune-Mediated Anemias in Ruminants and Horses

KATHY K. SEINO

Immune-Mediated Hemolytic Anemia

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Equine Infectious Anemia

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Acronyms and Abbreviations

AGID, agar gel immunodiffusion test; AIHA, autoimmune hemolytic anemia; CR1, complement receptor 1; CR3, complement receptor 3; EIA, equine infectious anemia; EIAV, equine infectious anemia virus; ELISA, enzyme linked immunosorbent assay; Fcγ receptor, fragment crystallizable gamma receptor; Hgb, hemoglobin; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; JFA, jaundiced foal agglutination (test); MAC, membrane-attack complex; NI, neonatal isoerythrolysis; PCV, packed cell volume; RBC, red blood cell; USDA, United States Department of Agriculture.

Immune-mediated hemolytic anemia (IMHA) is an accelerated destruction of red blood cells (RBCs) caused by antibody bound to erythrocytes. In comparison with dogs and cats, IMHA in horses and ruminants appears to have no breed or gender predilection and occurs less frequently, but is still an important cause of anemia.^{16,23,39} IMHA may be primary or secondary in nature. Primary IMHA occurs when antibodies are formed against the normal RBC surface antigens. This can occur with incompatible blood transfusions, in neonatal isoerythrolysis, or with idiopathic autoimmune hemolytic anemia. Secondary IMHA occurs when antibodies are formed against substances that abnormally coat the RBC.^{23,35} Reported cases of IMHA in horses are most commonly secondary (Table 35.1) rather than primary, with 15–26% associated with clostridial infections.³⁹ IMHA is a rare cause of anemia in cattle, with nine reported cases. Most affected cattle were young animals.

This chapter provides an overview of the major causes of immune mediated anemia in ruminants and horses, focusing on immune mediated hemolytic diseases, neonatal isoerythrolysis and equine infectious anemia.

IMMUNE-MEDIATED HEMOLYTIC ANEMIA

Primary IMHA (i.e. AIHA) resulting from an idiopathic loss of self-tolerance to RBC surface antigens, is a rare condition in large animals.^{1,17} Secondary IMHA in horses and ruminants is associated with infection, neoplasia, and exposure to drugs or toxins. Drug-induced hemolytic anemia is a Type II hypersensitivity reaction in which destruction of RBC occurs by one of three mechanisms: (1) the drug and antibodies combine to activate complement and destroy bystander RBC; (2) the drug acts as a hapten, a molecule that is not

TABLE 35.1 Causes of Immune-Mediated Hemolytic Anemia (IMHA) in Horses and Ruminants

	Horses	Cattle
Primary IMHA		
Incompatible blood transfusion		
Neonatal isoerythrolysis	References 4, 9, 15, 18	Reference 34
Autoimmune hemolytic anemia (idiopathic)	References 2, 16, 21, 32	References 5, 7, 11, 37
Secondary IMHA		
Infectious	Equine infectious anemia ²⁰ <i>Clostridium perfringens</i> ^{27,39} <i>Clostridium septicum</i> ^{25,39} Injection abscesses ^{26,39} <i>Streptococcus</i> spp. ^{10,36}	<i>Salmonella dublin</i> ¹² Sarcocystis ⁸ Trypanosomiasis ⁶
Drugs/toxins	Penicillin ^{3,19,29} Sulfonamides ³⁵ Organophosphate ³³ Lymphosarcoma ²⁸	Polyvalent botulism vaccine ⁴¹
Neoplasia	Melanoma, other neoplasias ^{16,36}	
Other systemic diseases	Protein losing enteropathy ²² Purpura hemorrhagica ¹⁰	

inherently immunogenic but binds to a carrier protein and covalently binds to RBC; or (3) the drug modifies the RBC membrane such that antibody coats the RBC (see Chapters 14 and 16).³⁶ There have been a number of equine cases of IMHA associated with penicillin acting as a hapten.

RBC destruction secondary to infectious disease is also a Type II hypersensitivity reaction. Lipopolysaccharides of bacteria and hemagglutinin subunits of equine infectious anemia virus (EIAV), for example, bind to RBCs resulting in antibody coating and binding of C3 complement (see Chapter 32).^{20,36} Direct RBC membrane injury by bacterial toxin is thought to be a potential mechanism of *Clostridium* induced IMHA in horses.³⁹

Pathogenesis of Immune Mediated Hemolytic Anemia

Regardless of whether the process was primary or secondary in nature, destruction of RBCs in IMHA is due to the removal of the antibody-coated RBCs by macrophages, primarily in the spleen and liver (extravascular hemolysis) or less frequently mediated by complement (intravascular hemolysis). Specifically, RBC bound with IgG is cleared from circulation via cells of the fixed mononuclear phagocytic system expressing a specific Fcγ receptor and most predominantly in the spleen. Immunoglobulin-coated RBC can fix C3 and are cleared by macrophages with CR1 and CR3. Alternatively, some immunoglobulin bound to RBCs activates the entire complement cascade with formation of the membrane-attack complex (MAC) and resultant RBC intravascular lysis.¹⁷

IgA antibody has not yet been identified in horses as an agglutinin in IMHA due to limited ability to identify surface-bound IgA of horses. IgA is involved in the development of purpura hemorrhagica.^{10,40}

Autoantibodies are further classified as warm or cold reactive. Warm RBC autoantibodies react strongly at 37°C, are associated with severe anemia and are predominantly IgG. Cold RBC autoantibodies react strongly at 4°C and tend to be IgM class.¹ A special subset of cold autoantibodies that are active at 30°C are thought to cause a cryopathic or cold hemagglutinin disease, in which the IgM agglutinates in the peripheral circulation of the extremities (distal limbs, ears, etc.) and causes ischemic necrosis and gangrene. This form of IMHA has been reported in the Freisian heifers as a consequence of *Salmonella dublin* infection.¹² Cold RBC autoantibodies that react only at 10–15°C appear not to cause pathology as they have been demonstrated in the serum of healthy animals.

Clinical Features of Immune Mediated Hemolytic Anemia

Primary and secondary IMHA are clinically indistinguishable. Onset of hemolytic disease is variable depending on the rate of destruction, severity of anemia, and underlying disease process. Common signs include fever, depression, icterus, and hemoglobinuria. With severe, profound hemolysis (PCV < 15%) clinical signs reported include: tachycardia, tachypnea, cold extremities, pale mucous membranes, muscle weakness, and heart murmur. Some animals may have an apparent splenomegaly based on ultrasound and rectal palpation findings.

Diagnosis of Immune Mediated Hemolytic Anemia

Diagnosis of IMHA is based upon demonstration of multiple clinicopathologic criteria: (1) RBC morphology changes consistent with RBC destruction; (2) anemia; (3) autoagglutination; (4) a positive direct antiglobulin

result (i.e. Coombs' test) in a test validated for use with the horse or ruminant; and (5) elimination of other underlying causes of anemia.

Red blood cell morphology changes that have been described include marked poikilocytosis and echinocytes (type III > type II). Spherocytosis was described in a *Clostridium perfringens* type A-induced IMHA in a Warmblood gelding. *Clostridium perfringens* type A produces α -toxin, a phospholipase that alters RBC membrane phospholipids.³⁹

Anemia of IMHA is usually quite severe with PCVs of less than 20%, but is typically regenerative as erythropoiesis is not affected. Unlike blood-loss anemia, horses with hemolytic anemia may develop very low levels of reticulocytosis. Low levels were detected in one horse at 13 days after the onset of anemia with the use of an automated reticulocyte count using chromagen Oxazin 750 staining of RBC.³⁹

Microscopic evaluation of a blood smear may also reveal autoagglutination with IMHA. In horses, in which RBCs are characterized by rouleaux formation, true autoagglutination is difficult to appreciate. Persistent agglutination, after washing or dilution in saline, differentiates autoagglutination from rouleaux formation.^{1,23}

Confirmation of anti-RBC antibody is done by use of a direct antiglobulin (Coombs') test (see Chapter 140). The patient's washed RBCs are incubated with antiserum to equine IgG, IgM, and complement. Specific identification of antibody-coated RBCs can be done with direct immunofluorescence using flow cytometry. The direct immunofluorescence assay detected low levels of cell-bound antibody and is valuable in monitoring the response to therapy in regards to monitoring reduction of RBC-bound and platelet-bound IgG in horses.^{38,40}

Additional hematologic changes in IMHA include leukocytosis with neutrophilia, hyperfibrinogenemia, and hyperbilirubinemia associated with pigmenturia. Urinalysis may indicate hemoglobinuria.

Further work-up should focus on the identification of the underlying disease process. Rectal palpation, abdominocentesis, ultrasound examination should be conducted for the detection of intra-abdominal abscesses and neoplasia. Auscultation, thoracic radiographs, and ultrasonography of the chest should be done to determine lower respiratory disease or thoracic masses. Transtracheal washes and/or thoracocentesis may be needed. Endoscopy of the upper airways and guttural pouches is recommended to look for potential *Streptococcus* spp., and lymphadenopathy. Additional laboratory tests include serology for various infectious diseases, particularly for equine infectious anemia (EIA), blood culture for bacteremia, and culture of abscesses.

Differential diagnoses for anemia in adult horses include EIA, ehrlichiosis, babesiosis, blood loss, chronic disease, leptospirosis, and neoplasia. Anaplasmosis, eperythrozoonosis, theileriasis, and bacillary hemoglobinuria, are included in the differential list for ruminants. Lymphosarcoma has been associated with

IMHA in horses, dogs, and humans, but no association has been documented with cattle.²⁸

Treatment of Immune Mediated Hemolytic Anemia

Once an animal is diagnosed, therapy should include discontinuation of any drugs associated with IMHA. If a primary disease process or infection is involved, antimicrobial therapy may necessitate switching of the class of antibiotic drug being used. Additional appropriate treatments of the underlying disease process should be instituted.

Immunosuppressive doses of corticosteroids are indicated with the purpose of stabilizing RBCs, decreasing production of antibody, decreasing the avidity of the antibody for RBC, and decreasing the rate of clearance of antibody-coated RBC. It is important to keep in mind that corticosteroids may be less effective for treating IMHA with intravascular hemolysis mediated by IgM and complement.³⁶ Additionally, in horses chronically infected with EIA, steroid immunosuppression may result in recrudescence of the virus.^{20,23} Horses with suspected IMHA should test negative for EIA before initiation of therapy. Dexamethasone (0.1 mg/kg [range 0.05–0.8 mg/kg IV or IM once or twice daily]) is the most commonly used corticosteroid in horses. Once the PCV stabilizes at greater than 20%, tapering of the steroid dose to 0.01–0.02 mg/kg daily can be initiated. Alternatively, prednisolone (1 mg/kg IM twice daily) may be used. Monitoring of the PCV should be continued. Treatment may be necessary for several weeks. In cases that were non-responsive to corticosteroids, cyclophosphamide, azathioprine, or vincristine has been used.^{21,39} Blood transfusion is indicated if anemia becomes life threatening; however, compatible blood donors may be difficult to find and transfusion may exacerbate the hemolytic process. Plasmapheresis was utilized in the treatment of IMHA in one horse with a *Clostridium perfringens* infection.³⁹ Splenectomy is utilized as a treatment modality in refractive cases of IMHA in dogs, but has not been described in large animals.

Blood transfusion in cattle is difficult and not commonly used because blood groups are complex and difficult to match (see Chapters 98 and 139). The B blood group system in cattle contains more than 60 antigens and is inherited in combinations of phenogroups.³⁶ Successful blood transfusion and treatment of a year old Holstein heifer with IMHA has been described.¹¹

Prognosis for Immune Mediated Hemolytic Anemia

The overall prognosis for horses with IMHA is dependent on the underlying disease process. Horses that have multiple recurrences of hemolysis are a high risk for having neoplasia and should be carefully examined. Reports of IMHA in cattle are too limited to make a reliable estimation of prognosis.

NEONATAL ISOERYTHROLYSIS

Neonatal isoerythrolysis (NI) is an important cause of anemia in newborn foals and mules. The prevalence of NI varies among different breeds but has been reported to be 1% in Thoroughbreds and 2% in Standardbred horses.⁹ In mules (donkey sire/horse dam), the case rate is higher at 8–10% due to a unique donkey RBC antigen.¹⁸ In severe cases, NI can be life-threatening. Neonatal isoerythrolysis is rare in ruminants, but can occur in cattle vaccinated against anaplasmosis or babesiosis.^{24,34}

Pathogenesis of Neonatal Isoerythrolysis

Neonatal isoerythrolysis is caused by maternal alloantibodies directed against specific surface blood-group antigens on the affected foal's RBCs. Alloantibodies are produced after sensitization of the dam with incompatible blood group RBCs (i.e. leakage of blood across the placenta during pregnancy or at delivery, transfusion of mismatched blood). Alloantibody production in the mare may persist for years. Within the eight equine blood groups, 32 blood group antigens are present on RBCs. Although alloantibodies can occur with any blood group, Aa and Qa account for the majority of NI cases. Factors Ab, Ac, Db, Pa, Pb, Qc, and Ua also have been associated with NI in horses. Interestingly, anti-Ca antibody may be protective for NI as it is commonly present, but not associated with the occurrence of clinical disease.^{4,9,31} A recent publication described NI in two Friesian foals due to a non-specific hemolytic alloantibody acting as a heterophil antibody (an antibody that arises in response to a cross-reactive epitope originating from the environment such as food or bacteria), but further research is needed to substantiate this.⁹

Neonatal isoerythrolysis is an acquired immunologic hemolytic disease. IgG alloantibodies do not cross the placenta but are acquired by ingesting colostrum and are absorbed intact into the foal's circulation during the first hours after birth. If the foal inherits the RBC antigens (from the sire) that its mare lacks, the foal is at risk of developing NI. The maternal alloantibodies bind to neonatal RBC, causing primarily hemolysis and also hemagglutination with subsequent extravascular or intravascular hemolysis.⁴ Thrombocytopenia, presumably due to antiplatelet alloantibodies, has been reported in mule foals.^{4,18}

In cows, sensitization occurs with vaccination for anaplasmosis and babesiosis. These vaccines contain large quantities of RBCs obtained from infected calves and induce antibodies against the A and F blood systems. If the sensitized cow is mated to a sire with these blood groups, the calf may be at risk of developing NI.^{24,30,36}

Clinical Features of Neonatal Isoerythrolysis

Typically, foals and calves are normal at birth with signs developing as early as 5 hours after birth, but usually at 12–48 hours of age. Signs vary from subacute

to peracute depending on the degree of anemia and rate of RBC destruction. Foals may present with lethargy, weakness, tachypnea, tachycardia, pale mucous membrane, icterus, pigmenturia, and hemodynamic shock. Death may occur if hemolysis is severe. Additionally, hypoxemia and tissue hypoxia may induce neurologic disorders, metabolic acidosis, and multiple organ failure.³¹ Extreme elevations of unconjugated bilirubin can lead to bilirubin encephalopathy or kernicterus in foals.¹³ Fulminant hemolysis in calves can trigger fatal disseminated intravascular coagulopathy.³⁶ Mule foals may develop concurrent thrombocytopenic purpura.^{4,31}

Diagnosis of Neonatal Isoerythrolysis

Neonatal isoerythrolysis should be considered in a newborn foal or calf presenting with weakness and icterus in the first few days of its life. It should be considered in a multiparous mare or mare with a previous history of foals with NI, but should also be considered even in a maiden mare. It should be considered in a cow vaccinated for anaplasma or babesia.

Anemia is the primary clinicopathologic finding in neonatal foals or calves with NI, with the PCV typically <20%. A decrease in the normal 3:1 ratio of PCV to hemoglobin may be noted due to the presence of free hemoglobin (Hgb) in serum. Other clinicopathologic findings include: hemoglobinemia and hemoglobinuria due to intravascular hemolysis and hyperbilirubinemia (20–40 mg/dL). If toxic hepatopathy occurs, liver enzymes, bile acids, and blood ammonia levels may be elevated.^{4,23} In a recent retrospective study of NI, four out of 15 foals with NI had failure of passive transfer.⁴

Diagnosis of NI requires demonstration of maternal alloantibody binding to foal RBCs. Lytic assays are considered more accurate as alloantibodies act more strongly as hemolysins. Agglutination tests may be hindered by rouleaux formation of equine blood. Hemolytic assays involve the mare's serum mixed with foal RBCs and complement (pooled rabbit serum). If the serum contains alloantibodies, hemolysis results in pink/red supernatant and decreased RBC mass after centrifugation.^{1,23} Similar hemolytic assays with appropriate bovine reagents can be performed in calves.³⁶

The jaundiced foal agglutination (JFA) test consists of serial dilutions of the mare's colostrum in the presence of 5% RBC suspension from the foal. Agglutination is the end-point of a positive test result (agglutination at dilutions of 1:16 or greater are considered significant). The JFA test is reported to correlate well with the hemolytic test and does not require an exogenous source of complement.³¹ The Coombs' test can be used to demonstrate the presence of antibodies or complement bound to foal RBC, but is not specific for the diagnosis of NI.

Differential diagnosis of NI in foals presenting with weakness and icterus include septicemia, hypoxic-ischemic encephalopathy, equine herpesvirus-1, and leptospirosis. Potential causes of anemia in neonates include hemothorax, rib fracture, umbilical bleeding, and external hemorrhage.

Prevention and Treatment of Neonatal Isoerythrolysis

Prevention of NI is twofold: (1) determining whether the foal is at risk, and (2) protecting the foal from exposure to alloantibody. Determination of risk requires blood typing of the mare before parturition. Mares that test negative for Aa, Qa, Ua, or Pa should be considered at risk for making alloantibodies when exposed to antigen. Stallions bred to Qa/Aa negative mares can also be blood typed to determine if they are Qa/Aa positive. Alternatively, the mare's serum can be screened for the presence of anti-RBC antibodies within 30 days before parturition using a panel of blood-typed RBCs or tested against the sire's RBCs. Before the foal is allowed to nurse, either a hemolytic test or JFA test should be performed.^{4,23,31} In calves, screening of serum and colostrum of vaccinated cows with a saline agglutination test has been reported to be helpful in predicting the occurrence of NI.¹⁴

If the mare's colostrum has been determined to contain alloantibodies and the foal is positive for the specific antigen, the foal should be muzzled and not allowed to nurse for the first 30–48 hours postpartum. The mare's udder should be stripped regularly during this time to remove all colostrum and maintain milk production. Colostrum from an alternative source should be administered to the foal.

Foals that are diagnosed with NI should be immediately prevented from further exposure. Foals with mild anemia may only require exercise restriction to prevent clinical signs of NI. Use of corticosteroids to reduce the rate of clearance of sensitized RBCs may be beneficial. Broad-spectrum antibiotics are recommended, particularly with the use of corticosteroids for the prevention of secondary sepsis. In foals with clinical signs of NI, supportive therapy with oxygen insufflation and/or oxyglobin therapy may improve oxygen saturation. Weak and recumbent foals need supplemental nutrition, intravenous fluid therapy, and nursing care.^{4,31}

In cases of severe anemia a blood transfusion is needed. In addition to clinical signs (weakness, acidosis, tachypnea), parameters indicative of blood transfusion include a PCV of <10–15%, RBC count <3 × 10⁶ cells/μL, and Hgb concentration ≤5 g/dL. The choice of donor is critical as the donor RBCs must be free of the specific reactive antigen. Donor whole blood should be from an Aa/Qa negative universal blood donor. Suitable donors should be crossmatched (donor RBCs with mare or foal serum). The optimal choice for donor is the mare, but the mare's RBCs need to be washed to remove alloantibody-containing plasma. The foal's sire is the least desirable donor as it has the same RBC antigenic factors as the foal. In the case of mule foals, because the offending antigen is the unique donkey antigen, RBCs from any horse could potentially be used for transfusion. Transfused RBCs have a 2–4 day lifespan in circulation. One to four liters of whole or washed RBCs is sufficient to induce clinical improvement. Depending on the foal's bone marrow response to anemia or continued hemolysis, additional transfusions may be needed.^{1,4,31}

Prognosis of Neonatal Isoerythrolysis

Prognosis for NI depends on the degree of alloantibody antigenicity. Cases with acute anemia, hypoxemia, and onset of seizures have a guarded prognosis. Foals that continue to have hemolysis after transfusion develop secondary degenerative hepathopathy, or signs of kernicterus have a grave prognosis.

EQUINE INFECTIOUS ANEMIA

Equine infectious anemia (EIA) is an important and reportable disease of horses and is characterized by IMHA. Horses of all ages and breeds are susceptible to infection with equine infectious anemia virus (EIAV). Despite the development of a sensitive and specific Federal regulatory test (Coggins test) and institution of regulatory guidelines for infected horses, EIA is still present in the United States, with outbreaks occurring sporadically throughout the country, but is most prevalent in the southeast.²⁰

Pathogenesis of Equine Infectious Anemia

Equine infectious anemia is caused by a non-oncogenic retrovirus of the *Lentivirus* genus that infects macrophages. Natural infection occurs from the transmission of infected blood through the interrupted feeding of hematophagous arthropods (primarily *Tabanus* spp. and *Chrysops* spp.). Upon infection, the virus multiplies in the host macrophages, incorporates into the genome, and persistently infects the host. Viral replication has been found to occur at all times even during subclinical phases at reduced rates.²⁰

Clinical Features of Equine Infectious Anemia

Three clinical forms of EIAV disease have been described in and include: acute, subacute to chronic, and chronic inapparent (carrier). In acute infection, the virus invades the host macrophages and undergoes massive replication. Clinical signs of acute EIA are seen within 7–30 days after exposure and manifests as fever, depression, anorexia, and mucosal petechiae. Thrombocytopenia is common during initial infection and leucopenia with lymphocytosis and monocytosis also may occur. Anemia is not seen in this stage.^{20,23}

In the subacute to chronic form of EIA disease, horses infected for longer than 30 days develop signs of weight loss, edema of the limbs and ventral abdomen, cyclical fevers, anemia, and icterus. Horses also can develop ataxia, abortion, colic, liver disease, and renal disease associated with viral infection. During the subacute and chronic form of disease, the PCV, RBC count, and Hgb levels decrease with hemolysis. Hemolysis is thought to occur from immune mediated processes wherein the hemagglutinin subunit of EIA virus binds to RBCs, causing the coating of RBC with antibody and subsequent binding of C3 complement.²⁰ The Coombs' test is positive during these periods. Increased liver enzymes

and proteinuria also may be seen. With infection of host macrophages, viral proteins induce strong humoral and cell-mediated immune responses, with most horses developing detectable serologic responses by 16–42 days post-infection. Continued viral production results in lymphoreticular hyperplasia and hypergammaglobulinemia.

Horses may succumb to infection either in the acute or subacute to chronic stages of disease, but most recover. Some horses have periodic recrudescence of the virus during periods of stress or treatment with corticosteroids, but a majority of horses remain clinically inapparent carriers of EIAV. No hematological abnormalities are detected in these horses.

Diagnosis of Equine Infectious Anemia

The Coggins test is still primarily utilized to diagnose EIA. The Coggins test is an agar gel immunodiffusion (AGID) test and detects serum antibodies against the EIAV gag p26 protein. The test is sensitive and specific but may provide false-negative results during the acute stage of EIA infection. Foals that have ingested colostrum from infected dams may have a false positive test. Repeat testing of suspected horses after 10–14 days and of suspected foals after 6 months of age (as maternal antibody levels decrease) is advocated. Three other tests have been approved by the USDA. These tests provide for more rapid testing and are more sensitive than the AGID, but are less specific and should be confirmed with the AGID.²⁰

Treatment and Prevention of Equine Infectious Anemia

There is no treatment for EIAV infection. Individual states and federal government require the humane destruction and/or quarantine of EIAV infected horses. State regulatory guidelines vary, but a physical quarantine of a 200-yard distance between affected horses and non-infected horses is required.

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Anemias Associated with Oxidative Injury

MICHEL DESNOYERS

Pathophysiology of Oxidative Injury

Increased Susceptibility of Feline Hemoglobin

Causes of Oxidative Injury in Domestic Species

Feline Species

Acetaminophen (paracetamol)

Diabetes mellitus

Propylene glycol

Hyperthyroidism

Lymphoma

Phenazopyridine

Methylene blue

Benzocaine

Onions

Canine Species

Onions/garlic/Chinese chive

Acetaminophen/paracetamol

Skunk musk

Zinc

Naphthalene

Diabetes mellitus

T-cell lymphoma

Vitamin K and vitamin K antagonists

Benzocaine

Equine Species

Red maple leaves

Garlic

Phenothiazine

Lymphoma

Bovine/Ovine Species

Selenium/copper deficiency

Chronic copper intoxication

Brassica

Onions

Oak leaves and shrubs

Acronyms and Abbreviations

FDA, Food and Drug Administration; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; HB, Heinz body; HK, high-potassium breeds; MB, methylene blue; NAPQI, *N*-acetyl-*para*-benzoquinoneimine; PAP, *para*-aminophenol; PDB, paradichlorobenzene; PG, propylene glycol; RBC, red blood cell; SAmE, *S*-adenosylmethionine; SMCO, *S*-methylcysteine sulfoxide; SOD, superoxide dismutase.

Red blood cells (RBCs) are highly specialized cells that perform the critical function as an oxygen carrier in a wide range of environments. They are continuously exposed to oxidant stress under both physiologic and pathologic conditions. Because circulating mature RBCs cannot synthesize new proteins or replace cellular components *de novo*, they are limited in their ability to respond to these stresses and may become irreversibly damaged. The most common consequence of oxidant injury to the red blood cell is the formation of Heinz bodies (HBs), eccentrocytes, and methemoglobin.

In 1890, Heinz first described round structures protruding from the surface of RBCs of humans and animals exposed to certain types of coal-tar drugs. These structures, which were associated with RBC destruction, were subsequently called Heinz bodies (HBs) and can

be found on the surface of RBCs in several species secondary to oxidative injury. Heinz bodies are formed by denatured clumps of precipitated hemoglobin on the inner surface of the RBC (Fig. 36.1). These structures can more easily be seen with new methylene blue (NMB) staining (Fig. 36.2).

Eccentrocytes (sometimes called erythrocyte hemighosts) are erythrocytes characterized by the concentration of hemoglobin on one side of the cell leaving a pale or clear portion on the other side (see Chapter 23).¹¹ Eccentrocytes are probably formed as a result of direct damage to the RBC membrane with adhesion of the opposing faces of the membrane⁵ (Fig. 36.3).

Methemoglobin is formed by the oxidation of ferrous iron (Fe²⁺) to ferric iron (Fe³⁺) in the heme groups and can be seen in combination with HBs and eccentrocytes (see Chapter 21).

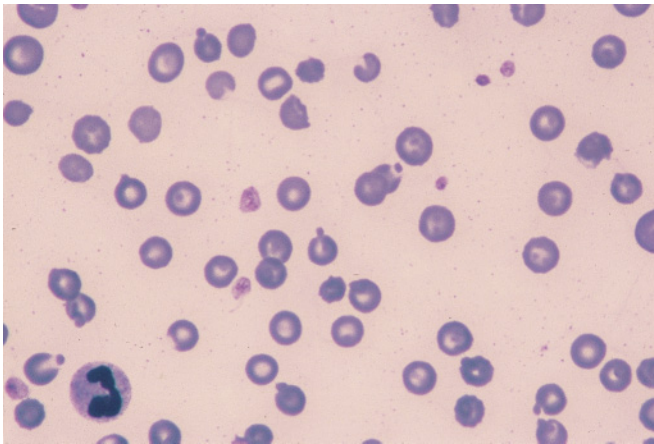


FIGURE 36.1 Heinz bodies with modified Wright-Giemsa stain: Heinz bodies are protuberances on the surface of RBCs. Original magnification 500 \times .

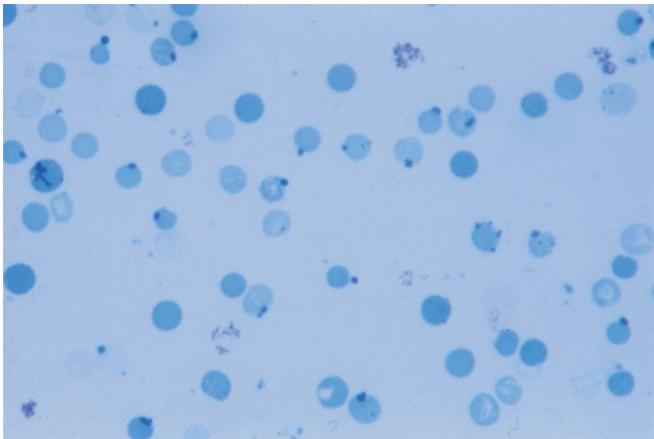


FIGURE 36.2 Heinz bodies with new methylene blue stain: Heinz bodies are dark round protuberances on the surface of erythrocytes. Original magnification 500 \times .

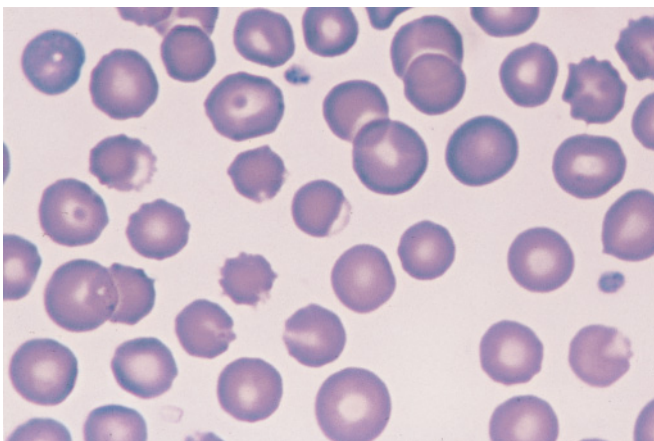


FIGURE 36.3 One eccentrocyte is present in the center and is characterized by hemoglobin concentrated on one side of the RBC, leaving a pale area at the other extremity. Modified Wright-Giemsa stain. Original magnification 500 \times .

PATHOPHYSIOLOGY OF OXIDATIVE INJURY

Oxygen carried by the RBC is a strong oxidant since it can generate highly reactive derivatives such as superoxide free radical (O_2^-), hydrogen peroxide (H_2O_2) and, by reacting with iron, it forms hydroxyl radical (OH^\bullet). These oxidants are constantly produced and RBCs have several mechanisms to prevent oxidation and damage to the hemoglobin (see Chapter 21). The enzymes superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx) and reduced glutathione (GSH) protect RBCs against oxidative damage.¹⁷ When these systems are overwhelmed, HB or eccentrocyte formation occurs: oxidation of the sulfhydryl groups on globin chains results in formation of HB whereas direct oxidative damage of the RBC membrane results in eccentrocyte formation.

INCREASED SUSCEPTIBILITY OF FELINE HEMOGLOBIN

Feline hemoglobin is more susceptible to oxidative damage than that of other mammalian species: this is due to the presence of eight reactive and fragile sulfhydryl groups on the molecule (instead of two strong ones in other species) and the easy dissociation of hemoglobin from a tetramer to a dimer form.⁷

The feline spleen is non-sinusoidal and inefficient at removing HBs from RBCs because of the large pores in splenic pulp venules which allow RBCs to enter without deformation. This explains why low numbers of HBs may be present in normal cats.⁷

Because RBCs may circulate relatively unimpeded in the spleen, splenic destruction of RBCs is not the major mechanism for the development of anemia in cases of oxidative stress in cats. Thus, other mechanisms must be present, such as increased rigidity of the RBC membrane, abnormal surface charge distribution and protease-activated HB removal, resulting in decreased RBC survival.⁷ Binding of globin to cytoskeletal proteins may result in loss of membrane integrity, increased membrane proteolysis, and subsequent RBC lysis.

CAUSES OF OXIDATIVE INJURY IN DOMESTIC SPECIES

Feline Species

Acetaminophen (Paracetamol)

In most mammalian species, acetaminophen (paracetamol in Europe) is primarily transformed to nontoxic products by the liver via conjugation with glucuronic acid and, to a lesser degree, sulfates, followed by elimination by the kidneys. In humans, most cases of acetaminophen intoxication involve hepatotoxicity.²² Dogs and cats are unique because they tend to develop methemoglobinemia and oxidative hemolytic anemia with signs of cyanosis, depression, facial edema, dyspnea

and vomiting occurring 2–4 hours post-intoxication.²² Oxidative hemolytic anemia is usually seen 48–72 hours post-intoxication whereas methemoglobinemia can occur as soon as 2–4 hours after ingestion.²²

In most species, acetaminophen is metabolized in the liver through sulfation and glucuronidation. A small percentage of acetaminophen is oxidized by cytochrome P450 to *N*-acetyl-*para*-benzoquinoneimine (NAPQI).² However, at toxic doses, both the sulfation and glucuronidation pathways become saturated and NAPQI production increases.²² If GSH is depleted to less than 20% of normal concentrations, NAPQI binds to cysteine groups on hepatocellular proteins, resulting in cellular death and centrilobular necrosis.²

Until recently, it was believed that a similar mechanism was also responsible for oxidative injury to RBCs, with NAPQI being the culprit. For instance, it was thought that cats produce more NAPQI than other species because they have limited acetaminophen glucuronidation;⁹ however, feline acetaminophen metabolism is primarily by sulfation, with glucuronidation playing a minimal role (less than 1.5%).²² Moreover, NAPQI is not likely produced by RBCs since they lack cytochrome P450 enzymes and NAPQI is not released in circulation by hepatocytes.²² Finally, NAPQI does not chemically react with oxyhemoglobin in a redox cycle.²²

Recently, it has been found that a minor acetaminophen metabolite, *para*-aminophenol (PAP) can react with oxyhemoglobin in a redox fashion.²² PAP is produced by deacetylation of acetaminophen by hepatic microsomal carboxyesterases.²² In most species, *N*-acetylation of drugs is catalyzed by two enzymes: *N*-acetyltransferase-1 and *N*-acetyltransferase-2. However, cats have only *N*-acetyltransferase-1 and dogs have no *N*-acetyltransferase enzymes. Therefore, dogs and cats are deficient in *N*-acetylation activity. This may result in greater quantities of PAP released into the circulation by the liver, with PAP reacting in a redox fashion with oxyhemoglobin. Moreover, canine and feline RBCs have low methemoglobin reductase activity²² and this limits the ability to efficiently reduce the methemoglobin produced by co-oxidation.²² Both mechanisms are probably responsible for the sensitivity of RBCs to acetaminophen in canine and feline species.

There is no safe dose of acetaminophen for cats. Toxic doses as low as 10mg/kg of body weight have resulted in death in cats.

Diabetes Mellitus

Oxygen-radical mediated damage has been recognized in both humans and animals with diabetes mellitus. Oxidative damage is caused by the formation of radicals secondary to glucose autooxidation, compromised antioxidant systems, oxidized plasma lipoproteins, alterations in inflammatory mediators as well as changes in antioxidant nutrient status.⁸ Cats with ketoacidosis can have significantly more HBs than nonketotic animals.⁸ The percentage of HBs in diabetic cats seems to be directly correlated with levels of plasma β -

hydroxybutyrate and inversely correlated with RBC glutathione concentrations.⁸

Cats with diabetes mellitus may become severely hypophosphatemic. Hypophosphatemia may impair phosphorylation of glucose to glucose-6-phosphate, preventing glucose entry into the pentose monophosphate shunt and thus impair formation of GSH: decreased GSH could result in increased HB formation.¹

Propylene Glycol

Propylene glycol (PG) itself is non-toxic to RBCs and a toxic metabolic intermediate may be required.⁷ Propylene glycol at a concentration varying between 6% and 13% was used several years ago in semi-moist food. Propylene glycol resulted in the formation of HBs, reticulocytosis, and decreased RBC lifespan. Despite formation of HBs and decreased RBC half-life, anemia is either not present or is minimal.¹⁸ Despite the absence of anemia in the majority of cats, the presence of oxidative injury to RBCs pushed the Food and Drug Administration's (FDA's) Center for Veterinary Medicine to prohibit the use of PG in cat food.

Hyperthyroidism

To our knowledge, only one study has looked at the association between hyperthyroidism and development of HBs in cats.⁶ In this study 15 out of 21 cats with hyperthyroidism had an increased percentage of HBs and their presence usually did not result in overt anemia.

Lymphoma

One study investigated the relationship between lymphoma and HB formation.⁶ In this study, 15 out of 17 cats with lymphoma had increased numbers of HBs. Mechanisms for HB formation are unclear but oxidative stress on bone marrow precursor cells may result in abnormal or damaged hemoglobin increasing the risk of HB formation.

Phenazopyridine

In cats, use of phenazopyridine (Pyridium®), a medication used for the treatment of urinary tract infections in humans,¹³ alone or in combination with other drugs, is contraindicated because of the high risk of dose-related methemoglobinemia and HB formation.¹⁵

Methylene Blue

Methylene blue (MB) is used in veterinary medicine either as a treatment for methemoglobinemia or less commonly as a urinary antiseptic.³³ In the treatment of methemoglobinemia, MB reacts with methemoglobin and reduces it to hemoglobin.³² Recent studies demonstrated that intravenous administration of single or limited doses of MB to treat methemoglobinemia did

not result in anemia despite an increase in the number of HB-containing RBCs.³²

Benzocaine

Benzocaine is an ethyl ester of *p*-aminobenzoic acid that is used as a local anesthetic in several over-the-counter medications (ex: Lanacane®). To our knowledge, only one case of benzocaine intoxication has been reported in cats.³⁷

Onions

Onions (*Allium cepa*) are considered to cause oxidative damage through thiosulfate compounds that form disulfide bonds with hemoglobin or GSH.³⁹ Onion intoxication is far less common in cats than dogs, probably due to the finicky eating nature of cats. However, baby food, commonly used in sick anorexic cats, may contain onion powder causing HB formation and sometimes anemia. The rate and degree of HB formation varies depending on the concentration of onion powder in the baby food.³⁰

Canine Species

Onions/Garlic/Chinese Chive

All types of onions (i.e. raw, cooked, or dehydrated) can be toxic to dogs.¹⁹ In onions, *n*-propyl disulfide, sodium *n*-propyl thiosulfate, sodium *trans*-1-propenyl thiosulfate and sodium *cis*-1-propenyl thiosulfate are thought to be the agents responsible for oxidative injury.^{38,40} Ingestion of garlic (*Allium sativum*) and Chinese chive (*Allium tuberosum*) can also result in oxidative injury.⁴⁰ The oxidative agent present in garlic is sodium 2-propenyl thiosulfate.⁴⁰ In cases of natural intoxication with garlic and/or Chinese chive, eccentrocytosis seems a more prominent feature of anemia than HB formation.⁴⁰ In dogs, there seems to be a correlation between the animal's weight and the development of HB in cases of onion/garlic/Chinese chive intoxication, as HB anemia is mostly seen in small breeds. So-called high potassium (HK) breeds (e.g. Akitas, Japanese mongrels, Shibas, Jindos, and Chinese Shar-Peis) are more susceptible to onion-induced oxidative damage than non-HK dogs.³⁸

Acetaminophen (Paracetamol)

Acetaminophen is a very popular over-the-counter analgesic and antipyretic drug and can result in both hepatic damage (zone-3 hepatocellular necrosis) and oxidative injury (eccentrocytes, HBs, and methemoglobinemia) in dogs. The recommended dose of acetaminophen is 15 mg/kg body weight per os q8 hours. Toxic dosage in dogs is around 200 mg/kg body weight.³⁴ Clinical signs may include tachycardia, tachypnea, blue-gray or pale brown mucous membranes, facial edema as well as brown urine and blood.²⁵ The mechanisms for RBC oxidative damage are discussed in the feline section.

Skunk Musk

Spraying with skunk musk is a rare cause of HB anemia and methemoglobinemia in dogs.⁴¹ Skunk spray contains thiols which may potentially react with oxyhemoglobin, forming methemoglobin thiol radicals and hydrogen peroxide.⁴¹ Both thiol radicals and hydrogen peroxide are highly reactive substances that may react with sulfhydryl groups on hemoglobin, resulting in the formation of HBs.

Zinc

Most cases of hemolytic anemia associated with acute or subacute exposure to zinc are related to ingestion of zinc-containing material, with the three most common sources being skin ointment (more than 100 products, including Desenex®), pennies minted in the USA after 1982, and metallic objects such as toys or nuts coated with zinc, which may be present in carrying cages.^{14,19} American pennies are composed of 98% zinc, each penny containing approximately 2,440 mg of elemental zinc.^{14,18} Most dogs with zinc intoxication are young (less than 2 years of age), since younger animals are more likely to ingest foreign objects than older animals. In one study,¹⁴ smaller dogs (mean weight 6 kg) were more likely to be intoxicated than larger dogs: smaller dogs have smaller pylori, making it more difficult for objects to pass from the stomach and allowing more time for erosion by gastric acid, freeing more zinc for absorption. A dose-related effect may also be involved.¹⁴ The pathophysiology of zinc-induced hemolysis in veterinary medicine is not completely understood but inhibition of GR and enzymes of the hexose-monophosphate pathway probably plays a role. Not all dogs with zinc intoxication develop RBC oxidative injury with formation of HBs or eccentrocytes.¹⁴

Naphthalene

Naphthalene, derived from coal tar, is one of two ingredients that can be found in mothballs, the other one being paradichlorobenzene (PDB).¹⁰ Naphthalene can also be found in other products, such as driveway sealants and toilet bowl deodorizers. Presently, PDB is more common than naphthalene in mothballs. Naphthalene is metabolized by the liver to α -naphthol, β -naphthol, α -naphthol-quinone and β -naphtholquinone.¹⁰ The metabolite responsible for the hemolytic crisis and HB anemia is α -naphthol.

Naphthalene intoxication seems relatively common in human medicine but, to our knowledge, is rare in veterinary medicine with only one case reported in a dog.¹⁰

Diabetes Mellitus

Pathophysiology and mechanisms for oxidative injury to RBCs in dogs with diabetes mellitus are similar to those in cats. Similar to cats, dogs with ketoacidotic

diabetes mellitus are at greater risk for oxidative damage than non-ketoacidotic animals.⁵

T Cell Lymphoma

In a retrospective study, several dogs with T cell lymphoma had evidence of eccentrocytes indicative of oxidative injury to RBCs. This is similar to cats, where several animals with lymphoma developed HBs.⁶ The mechanism for oxidative injury is unclear.⁵

Vitamin K and Vitamin K Antagonists

Both vitamin K administration and vitamin K antagonist intoxication have been reported as causes of oxidative injury in dogs.^{5,11} In cases of vitamin K administration, both vitamin K₃ and vitamin K₁ may result in HB formation, with vitamin K₃ being more toxic than vitamin K₁.¹¹ Vitamin K₃ reacts with hemoglobin and oxidizes oxyhemoglobin to methemoglobin with production of an intermediate, the semiquinone radical.¹¹ Semiquinone can combine with O₂ to produce H₂O₂ and contribute to RBC membrane damage. In the study of Caldin et al.,⁵ more dogs that were intoxicated with vitamin K antagonists had eccentrocytosis than dogs with vitamin K administration. It is therefore possible that HBs present in dogs treated with vitamin K were present prior to vitamin K administration. Different explanations are possible for oxidative injury in cases of vitamin K antagonist intoxication: vitamin K antagonists themselves may have oxidative potential. It is also possible that an additive to the drug has oxidant properties. Finally, pathological abnormalities caused by vitamin K antagonism (for example, acidosis caused by blood loss) may lead to oxidative damage.⁵ In conclusion, both vitamin K administration and vitamin K antagonist intoxication can cause oxidative damage to RBCs, resulting in the formation of HBs and/or eccentrocytes.

Benzocaine

Benzocaine is a local anesthetic that can be used as a topical anesthetic. Rare cases of HB anemia/hemoglobinemia have been reported in dogs secondary to benzocaine-containing products.^{16,19}

Equine Species

Red Maple Leaves

Dried red maple (*Acer rubrum*) leaves can induce toxicosis with oxidative injury to the RBC membrane and hemoglobin, resulting in hemolytic anemia characterized by HBs, eccentrocytes, and/or methemoglobinemia.³ There does not seem to be a sex, breed, or age predisposition for this condition and most cases seem to occur either during the summer or fall.³ Clinical signs may include tachypnea, tachycardia, icterus, cyanosis, scleral petechiation, and brownish discoloration of urine and blood. Signs appear as soon as 24 hours after ingestion with a peak 72 hours after ingestion.¹²

Leaves are toxic at a dosage of 1.5g/kg of body weight or more.¹² Gallic acid, present in red maple leaves, is often implicated in the oxidative damage of equine hemoglobin but experiments suggest that another still unidentified agent in red maple leaves must be present in combination with gallic acid to enhance methemoglobin formation.³ These toxins are not destroyed by prolonged storage or freezing.¹² In one study, looking at 32 horses with red maple leaf intoxication, HBs or eccentrocytes were noted in only 33% of affected animals.³

Garlic

Garlic can be used as an antifungal ingredient in livestock feed in order to prevent mycotoxin contamination.²⁶ Horses fed freeze dried garlic at a dosage of 0.2 g/kg of body weight or more showed signs of oxidative injury to RBCs with decreased RBC parameters and presence of HBs: these changes occurred 4 days after initial feeding and were associated with sweating.²⁶ Complete recovery occurred within 5 weeks after cessation of garlic supplementation.

Phenothiazine

Phenothiazine was one of the first broad-spectrum veterinary anthelmintics. Presently it is used only in horses but is not recommended for use in pregnant mares because it can result in abortion. Its principal use is in mixtures with piperazine and in small daily doses to inhibit egg-laying by resident intestinal parasites.⁴ Phenothiazine is degraded in the gastrointestinal tract to phenothiazine disulfide, an oxidative metabolite.²³ Clinical signs associated with intoxication may include tachycardia, anorexia, dullness, pale mucous membranes and opaque brown urine. The toxic dosage seems to be 30g or more. Anemia usually develops over a period of 6–13 days after administration.

Lymphoma

Anemia caused by oxidative injury is an uncommon sequela of lymphoma in horses. In a recent review of equine lymphoma, none of the anemic horses had evidence of oxidative injury but the majority had evidence of agglutination, suggesting an immune-mediated component.²¹ To our knowledge, there is only one case report of HB anemia associated with lymphoma in a Quarter Horse filly.³¹

Bovine/Ovine Species

Selenium/Copper Deficiency

Selenium deficiency with or without concurrent copper deficiency can be seen in cattle and sheep.^{24,35} In cattle, selenium deficiency can lead to a marked decrease in the selenium-containing enzyme GPx, whereas copper deficiency may result in HB anemia because superoxide

dismutase (SOD) is a cupro-enzyme. SOD protects RBCs from oxidative damage caused by accumulation of superoxide.³⁶ If both deficiencies are present at the same time, a cumulative effect may be present because both enzymes are required to detoxify superoxide free radicals.

Chronic Copper Intoxication

In sheep, copper toxicosis may also result in hemolytic anemia due to oxidative damage. Sheep are more susceptible to copper intoxication than other species because hepatocytes store copper and biliary excretion is limited. When liver copper increases there is increased hepatocyte turnover enhancing both cell division and apoptosis.²⁰ When the rate of hepatocyte apoptosis and loss exceed replacement, copper is released into circulation, resulting in intravascular hemolysis. Copper intoxication is also possible in cattle, but is less common than in sheep.³⁶

Brassica

Forage brassicas (cabbage, kale, forage rape) constitute a feed with high nutritional value, allowing a reduction in the purchase of concentrates.²⁸ However, brassicas contain sulfur compounds such as S-methylcysteine sulfoxide (SMCO) which is first converted in the rumen to thiosulfonate. Thiosulfonate is further metabolized to n-propyl disulfides, dipropyl disulfides and dipropenyl disulfides. All three disulfides are oxidizing agents, with dipropenyl disulfides being the most powerful.

When cattle are fed brassica-type plants, HB numbers peak at 10 days to more than 4 weeks after the initiation of feeding. In extreme cases, the hematocrit may fall to 30–50% of initial values.²⁸ The severity and rapidity at which the anemia develops is proportional to the amount of *Brassica* in the diet. Total recovery is achieved when animals are completely removed from *Brassica* feeding. RBC parameters return to reference intervals 3–4 weeks after complete removal of the plants.

Onions

Onions, garlic and other plants of the *Allium* family contain SMCO. Cattle are less susceptible to onion toxicity than dogs and cats but are more susceptible than sheep and goats.²⁹ Ruminal fermentation of SMCO leads to the formation of oxidative metabolites.²⁹ A study looking at onion feeding in cattle showed that feeding up to 25% cull onions on a dry-matter basis resulted in mild decreases in RBCs, hemoglobin, and packed cell volume as well as appearance of HBs in RBCs but did not result in clinical anemia.

Oak Leaves and Shrubs

Oak trees and shrubs are present in most countries, and ingestion of immature leaves or freshly fallen acorns is

the most common source of intoxication. Immature leaves and acorns are rich in tannic acid that is metabolized to pyrogallol and resorcinol.²⁷ Pyrogallol can trigger oxidation of hemoglobin to methemoglobin²⁷, resulting in oxidative damage to RBCs.

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Anemia of Inflammatory, Neoplastic, Renal, and Endocrine Diseases

MICHAEL M. FRY

Inflammation
Neoplasia

Renal Disease
Endocrine Disease

Acronyms and Abbreviations

CRF, chronic renal failure; DIC, disseminated intravascular coagulopathy; DM, diabetes mellitus; EPO, erythropoietin; ESA, erythropoiesis-stimulating agent; HSA, hemangiosarcoma; IL, interleukin; IMHA, immune-mediated hemolytic anemia; LEAP-1, liver-expressed antimicrobial peptide; RBC, red blood cell; rEPO, recombinant canine erythropoietin; rEPO, recombinant feline erythropoietin; rhEPO, recombinant human erythropoietin.

Many pathologic conditions may cause secondary anemia. This chapter will focus on inflammation, neoplasia, renal disease, and endocrine disease as causes of anemia. These categories are not mutually exclusive. Anemia in patients with these conditions is usually, but not necessarily, nonregenerative.

INFLAMMATION

For many years physicians and veterinarians have recognized that patients with infectious or inflammatory disease frequently develop anemia, and that this phenomenon is associated with altered iron homeostasis.¹³⁻¹⁶ The condition has been termed “anemia of chronic disease” or “anemia of inflammation” – the terminology is not consistent even in the contemporary literature. For the purposes of this chapter, it will be called “anemia of inflammation”. Affected individuals have some laboratory features of iron deficiency (hypoferrremia and decreased total iron binding capacity), but increased iron stores in the mononuclear phagocyte system – seemingly paradoxical findings that are consistent with functional iron deficiency. In contrast to patients with classical iron deficiency anemia, which in advanced cases is microcytic and hypochromic (see Chapter 26), patients with anemia of inflammation typically have normocytic, normochromic, nonregenerative anemia. This pattern is recognized in domestic animals in both research and clinical contexts. Experimentally

induced sterile inflammation in dogs and cats resulted in normocytic, normochromic anemia, hypoferrremia, and decreased total iron binding capacity.^{15,16,46} Experimental ehrlichial infection studies in dogs resulted in similar findings.⁵ Experimental infection of calves with *Psoroptes ovis* resulted in bone marrow myeloid hyperplasia, increased plasma fibrinogen concentration, and moderate nonregenerative anemia.⁴⁰

The veterinary clinical literature contains many references to anemia of chronic disease or anemia of inflammation, and it is generally accepted to be a common clinical entity.³⁴ However, the relationship between inflammation and anemia in animals with naturally occurring disease has received little attention.

A proposed evolutionary rationale for anemia of inflammation is that it results from an immunologic adaptation to limit microbial access to iron.^{13,17} The past decade has seen breakthroughs in the understanding of molecular mechanisms of anemia of inflammation. Many of the new insights involve hepcidin, a hormone now generally accepted as the key mediator of anemia of inflammation.¹⁷ Initially recognized as an antimicrobial peptide and referred to as liver-expressed antimicrobial peptide (LEAP-1), hepcidin was subsequently identified as an acute phase protein and a key inhibitory regulator of iron metabolism.^{17,22,33} It is synthesized mainly by hepatocytes – hence the name hepcidin – intimating both its tissue of origin and its direct antimicrobial effects. The bioactive form is a 25-amino acid

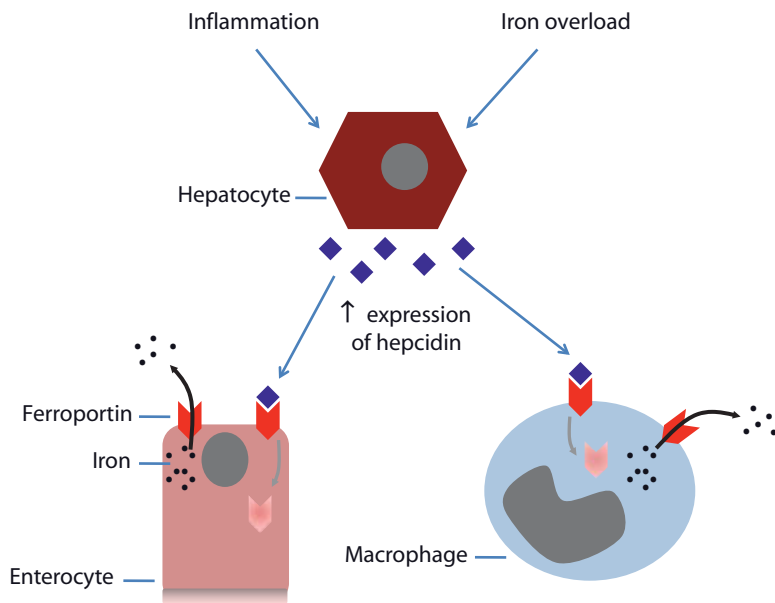


FIGURE 37.1 Hepcidin expression is increased by inflammation and iron overload. Hepcidin binds to ferroportin and causes its internalization and degradation, thus inhibiting efflux of iron into the plasma.

peptide that exerts its effects by binding to the cell surface iron efflux protein, ferroportin, and inducing its internalization and degradation.¹⁷ The effect of this interaction is to inhibit both absorption of dietary iron from intestinal epithelium and export of iron from macrophages and hepatocytes. Overexpression of hepcidin (for example by hepcidin-secreting tumors) results in iron deficiency anemia, while deficiency of hepcidin is a feature of most recognized forms of hereditary hemochromatosis.¹⁸

Hepcidin expression increases in response to inflammation and iron overload (Fig. 37.1).³² While interleukin-6 (IL-6) plays a central role in increasing hepcidin production, other cytokines, including interleukin-1 (IL-1), are also likely involved. Conversely, hepcidin expression decreases in response to anemia caused by hemorrhage or hemolysis, and in conditions associated with hypoxia, and iron deficiency.^{4,17}

Inflammation can contribute to anemia via mechanisms other than hepcidin-mediated disruption of normal iron handling. Studies have implicated inflammatory cytokines as inhibitors of erythropoiesis via direct toxic effects on erythroid precursors (e.g. via free radical formation or induction of apoptosis), decreased expression of hematopoietic factors including erythropoietin (EPO) and stem cell factor, and decreased expression of EPO receptors. Inflammation has also been shown to cause decreased red blood cell (RBC) survival in some species.^{45,46} These findings may not be equally applicable to all species: calves treated with endotoxin had a mean RBC lifespan slightly shorter than that of control animals, but the difference was not statistically significant.⁴² Some investigators have suggested that oxidants produced by activated neutrophils alter RBC surface antigenicity, which could in turn lead to accelerated destruction of RBCs by immune-mediated mechanisms (see Chapter 22).⁴⁴

NEOPLASIA

Anemia is common in humans with cancer, especially in cases of hematologic malignancies, and is recognized to have a negative impact on quality of life, prognosis, and response to chemoradiotherapy.¹⁹ Recognized causes of anemia in people with cancer include both disease- and treatment-related factors.¹⁹ Disease-related factors include acute or chronic hemorrhage, secondary immune-mediated hemolysis, impaired renal function resulting in decreased EPO production, nutritional deficiencies (folate, vitamin B₁₂, iron), and neoplastic infiltration of bone marrow. When not attributable to one of these causes, anemia in people with cancer is most likely anemia of inflammation, as described above.¹⁹ There is a high incidence of anemia as a complication of myelosuppressive chemotherapy in humans with cancer.¹⁹ Hemolytic anemia has also been reported secondary to anti-neoplastic therapy.¹⁹

The prevalence of anemia in the general population of animals with cancer is not well-characterized, but anemia certainly is recognized as a complication of cancer in veterinary medicine and is documented to be common in some types of cancer.^{1,10,29} The deleterious effects of anemia on quality of life in veterinary cancer patients have received little attention and need further investigation. As in people, recognized causes of anemia in animals with cancer include both disease- and treatment-related factors.²⁵ Disease-related mechanisms of anemia include hemorrhage, hemolysis, and decreased or ineffective erythropoiesis. Bleeding may occur directly from a neoplastic lesion, or distant sites due to defective hemostasis. Hemolysis may occur via phagocytosis of RBCs by neoplastic cells, secondary immune-mediated destruction, oxidative insult, or as mechanical damage (e.g. microangiopathic hemolysis). Decreased erythropoiesis may occur due to inflammation, space

occupation of the bone marrow by neoplastic infiltrates or fibrosis; elaboration of myelotoxic factors (e.g. estrogen); or increased apoptosis. Frequently the cause of anemia in animals with cancer is multifactorial. In principle, anemia can occur as a complication of virtually any type of neoplasia, but the risk is higher in certain types of malignancies. While most of the examples listed below are in dogs, the pathologic principles are not species-specific.

Primary or metastatic bone marrow neoplasia in animals can cause anemia by displacing normal hematopoietic tissue, inciting secondary myelofibrosis, and causing ineffective hematopoiesis due to increased apoptosis of hematopoietic cells.^{3,43}

Anemia due to hemorrhage is a frequent finding in some types of neoplasia in animals. For example, hemoperitoneum is a frequent complication of splenic hemangiosarcoma (HSA) in dogs.²⁰ Bleeding may be caused by rupture of the primary tumor, but exacerbated by other tumor-related pathology. Thrombocytopenia occurs in the majority of these cases and disseminated intravascular coagulopathy (DIC) is a frequent complication.²⁰ Anemia and clinical evidence of bleeding are frequent in dogs with gastrointestinal sarcomas and renal neoplasia. Hyperestrogenism-induced bone marrow hypoplasia is a recognized complication of canine Sertoli cell neoplasia.³⁹

Neoplasia can also cause anemia by hemolytic mechanisms. Canine hemophagocytic histiocytic sarcoma (see Chapter 73), as its name implies, is characterized by malignant histiocytes that are erythrophagic.²⁹ The large majority of affected dogs have Coombs'-negative regenerative anemia, and thrombocytopenia. Erythrophagocytosis is the major cause of anemia. Although bleeding is not a clinical characteristic of this disease, thrombocytopenia and other laboratory evidence suggest that hemorrhage may also play a role.

Neoplasia may cause immune-mediated or oxidative destruction of RBCs. Underlying neoplasia is a frequent differential diagnosis for immune-mediated hemolytic anemia (IMHA) in animals.²⁵ In a retrospective study on equine lymphoma, the majority of affected animals were anemic, and autoagglutination was a frequent finding.²⁸ Other mechanisms that may have contributed to the anemia in these horses include decreased erythropoiesis due to tumor myelophthisis or anemia of inflammation. Hemorrhage was not described in these horses; however, thrombocytopenia was noted in greater than one-third of cases. Lymphoma is also a frequent differential diagnosis in small animals with IMHA.³⁰ However, a causative relationship between anti-RBC antibodies and anemia has not been clearly shown.

An association between lymphoma and oxidative damage to RBCs, with or without anemia, has been documented in cats and dogs. A study in cats found Heinz bodies to occur significantly more often in animals with lymphoma (15 of 17 cases) than in a healthy control group.⁸ However, anemia was not a consistent finding in affected individuals. A recent retrospective study of canine eccentrocytosis found the

prevalence of the condition was only 1.4% (60 of 4,251 dogs), but every animal with T-cell lymphoma (5 of 5) had eccentrocytosis; however, information about the prevalence of anemia in these dogs was not provided.⁶

Many anti-neoplastic therapies are known to cause myelosuppression in humans and animals, and such treatment is likely to be a contributing factor to anemia in affected individuals. However, the impact of myelosuppression on RBCs is less severe than on other blood cell types because of the long RBC lifespan, and monitoring of hematologic toxicity of chemotherapy or radiotherapy typically focuses on neutrophils and platelets.²⁵ In the opinion of at least some veterinary oncologists, chemotherapy is more likely to cause treatment-limiting anemia as a result of gastrointestinal hemorrhage than as a result of myelosuppression (A LeBlanc, personal communication).

RENAL DISEASE

Nonregenerative anemia is an expected complication of chronic renal failure (CRF) in humans and in domestic animals, and is recognized to contribute to overall morbidity.¹¹ The major cause of anemia in patients with CRF is decreased production of EPO. Other contributing factors recognized in people include inflammation, hemorrhage, iron deficiency, decreased RBC survival due to metabolic insult or mechanical damage, and the anti-proliferative effects of uremic toxins.³¹ Concurrent disease may increase the risk of anemia in patients with renal disease (e.g. diabetes mellitus is a risk factor for development of anemia in human beings with impaired renal function).

The frequent occurrence of nonregenerative anemia in patients with CRF is well documented in the veterinary literature.¹² A recent report of juvenile nephropathy in 37 Boxer dogs found anemia to be a frequent finding (44% of cases), but did not characterize the anemia as regenerative or nonregenerative.⁷ Animals with CRF typically have inappropriately low plasma EPO concentrations. In a study of plasma EPO concentrations in dogs and cats, anemic animals without CRF had significantly increased plasma EPO concentrations compared to healthy controls, while a similar increase was not present in animals with CRF.³⁷ In cats there was no overlap in EPO concentrations between anemic animals with and without CRF; however, in dogs there was considerable overlap between these two disease groups. In a study of anemia of CRF in dogs, 12 of 17 patients had nonregenerative anemia, and there was a direct correlation between severity of azotemia and anemia.²¹ It is not clear how frequently or to what degree factors besides decreased EPO production contribute to development of anemia in animals with renal disease.

Recombinant EPO has been used extensively to treat anemia associated with CRF in humans and has been used to a lesser extent in dogs and cats. However, the use of such "erythropoiesis-stimulating agents" (ESAs)

is not without risk, as has been documented in the human literature, and the Food and Drug Administration recently ordered changes in the labeling of ESAs (<http://www.fda.gov/cder/drug/infopage/RHE/default.htm>; accessed September 2, 2008). Demonstrated or potential major risks of ESA therapy in humans include thromboembolism, promotion of tumor growth, and decreased survival. ESA therapy in people has also been shown to result in transient functional iron deficiency due to an imbalance between supply (available iron) and demand (increased erythropoiesis), a situation that can be remedied with intravenous iron therapy.

A potential side-effect of using recombinant human EPO (rhEPO) in animals is marked erythroid hypoplasia, the most severe manifestation being pure red cell aplasia (see Chapter 38). This is presumed to occur due to induction of antibodies that not only neutralize the recombinant EPO but are cross-reactive with the patient's endogenous EPO. This complication has been recognized in dogs, cats, and horses treated with rhEPO.³⁸ Species-specific recombinant canine (rcEPO) and feline EPO (rfEPO) have been developed; however, rfEPO resulted in development of red cell aplasia in some cats.³⁸ Neither rfEPO nor rcEPO is commercially available at the time of this writing.

ENDOCRINE DISEASE

Endocrinopathies are recognized to cause anemia in humans and animals. This section will focus on the relationship between anemia and several endocrine diseases that are well characterized in veterinary medicine: diabetes mellitus (DM), hypothyroidism, hyperthyroidism, and hypoadrenocorticism. Some of these conditions are associated with polyuria, and decreased plasma volume may mask the severity of anemia.²⁴

The etiology is multifactorial, but development of anemia in patients with DM is closely linked with renal disease. In humans with DM, anemia occurs with secondary renal disease and functional EPO deficiency. Various other factors may also contribute to the anemia.^{26,41}

Diabetes mellitus has been shown to cause increased Heinz body formation in cats, but it is not clear exactly how this relates to development of anemia (see Chapter 36). One study found Heinz bodies to occur significantly more often in cats with DM (19 of 21 cases) than in a healthy control group.⁸ Evidence presented in this study suggested a causal relationship to anemia. A subsequent study found that cats with diabetic ketoacidosis had more Heinz bodies than non-ketotic diabetic cats. However, a significant association between percentage of Heinz bodies and degree of anemia was not found.⁹ Heinz bodies were also detected in five cats with DM and hemolytic anemia associated with hypophosphatemia, but the hemolysis in these cats was presumed to be caused by hypophosphatemia.²

Nonregenerative anemia is a recognized complication of hypothyroidism in humans and animals, occurring in approximately one-third of cases in dogs.^{35,36}

Anemia develops primarily as a result of decreased erythropoiesis, with possible mechanisms including decreased EPO production, decreased responsiveness of developing erythroid cells to EPO, and direct effects of thyroid hormone on hematopoietic stem cells and erythroid progenitor cells.³⁶

Despite thyroid hormone effects in promoting erythrocyte production, anemia occurs in approximately 10–25% of people with hyperthyroidism.²³ Decreased erythrocyte survival and ineffective erythropoiesis have been suggested to play a role in development of anemia in humans with hyperthyroidism. A study in cats found Heinz bodies to occur significantly more frequently in animals with hyperthyroidism (15 of 21 cases) than in a healthy control group, but not all affected animals were anemic and the study did not demonstrate a causal relationship between Heinz bodies and increased erythrocyte destruction.⁸

Humans and animals with hypoadrenocorticism are frequently anemic, but the anemia may be masked by decreased plasma volume.^{24,27} The pathophysiology of anemia in hypoadrenocorticism is not well understood, but most likely involves decreased erythropoiesis, because glucocorticoids have been shown to interact with EPO and stem cell factor to promote erythropoiesis.²³ In a retrospective study of 225 dogs with hypoadrenocorticism, the prevalence of anemia was between 18% and 27%, depending on whether PCV or hemoglobin concentration, respectively, was used as an index of RBC mass. Blood loss, especially gastrointestinal hemorrhage, may also contribute to development of anemia in dogs with hypoadrenocorticism.²⁷

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Pure Red Cell Aplasia

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Pure Red Cell Aplasia in Dogs

Immune-mediated Pure Red Cell Aplasia

Definition and incidence

Clinical features

Laboratory features

Differential diagnosis

Therapy, course, and prognosis

Parvovirus Infection- or Vaccination-associated Pure Cell Aplasia

Pure Red Cell Aplasia Associated with an Immune Response to Recombinant Human Erythropoietin

Red Cell Aplasia Associated with Myelodysplastic Syndromes (see Chapter 66)

Congenital Red Cell Aplasia

Pure Red Cell Aplasia in Cats

Immune-mediated Pure Red Cell Aplasia

Definition and incidence

Clinical features

Laboratory features

Differential diagnosis

Therapy, course and prognosis

Feline-Leukemia Virus-induced Pure Red Cell Aplasia

Definition and pathogenesis

Clinical features

Laboratory features

Course and prognosis

Pure Red Cell Aplasia Associated with an Immune Response to Recombinant Human Erythropoietin

Red Cell Aplasia Associated with Myelodysplastic Syndromes (see Chapter 64)

Acronyms and Abbreviations

BFU-E, burst-forming unit-erythroid; CFU-E, colony-forming unit-erythroid; DNA, deoxyribonucleic acid; FeLV, feline leukemia virus; IgG, immunoglobulin G; IgM, immunoglobulin M; IMHA, immune-mediated hemolytic anemia; PCV, packed cell volume; PRCA, pure red cell aplasia; RBC, red blood cell.

Pure red cell aplasia (PRCA) defines a condition characterized by selective failure of erythropoiesis.^{10,17,18,21} The cardinal findings include severe anemia, reticulocytopenia, and absence or extreme depletion of erythroid precursors in bone marrow (Fig. 38.1).^{17,18,21} The anemia is normocytic and normochromic, and granulopoiesis and thrombopoiesis are unaffected. Pure red cell aplasia must be differentiated from aplastic anemia which, although the term has the same meaning, has been used to specifically denote pancytopenia of the bone marrow (see Chapter 39).⁵

Pure red cell aplasia occurs in both dogs and cats, and most frequently results from an acquired immune response that attacks early erythroid precursor cells in the bone marrow. This can occur as a primary immune-mediated disease process or as a sequela of treatment

with recombinant erythropoietin.^{19,20,29} Selective erythroid cell destruction or altered replication also can occur secondary to viral infections or modified live virus vaccination.^{2-4,8}

PURE RED CELL APLASIA IN DOGS

Immune-Mediated Pure Red Cell Aplasia

Definition and Incidence

Immune-mediated PRCA is considered to be a type of nonregenerative immune-mediated hemolytic anemia (IMHA).^{18,28} Other types of nonregenerative IMHA include IMHA with arrested maturation and IMHA

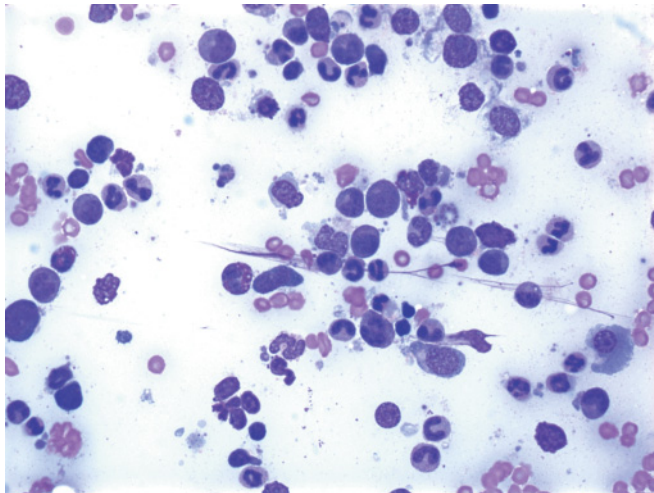


FIGURE 38.1 Bone marrow aspiration smear from a dog with immune-mediated pure red cell aplasia. Notice the presence of a normal population of granulocyte precursors but absence of erythroid precursor cells. Wright-Giemsa stain.

with erythroid hyperplasia (see Chapter 33). In one study of 82 dogs with primary nonregenerative IMHA, 33% had PRCA, 46% had nonregenerative IMHA with bone marrow erythroid hyperplasia, and 21% had nonregenerative IMHA with erythroid maturation arrest.²⁸ In another study, of 43 cases, 95% had maturation arrest or erythroid hyperplasia and only 5% had PRCA.¹⁸ The overall incidence of PRCA is unknown but PRCA was diagnosed in 3.4% of dog bone marrows in a retrospective study of clinical bone marrow reports at a veterinary teaching hospital.²⁵

The inclusion of PRCA as a subtype of nonregenerative IMHA is based on several observations. These include detection of serum antibodies that inhibit erythroid colony formation in vitro, positive direct Coombs' test results, and spherocytosis in some dogs.^{18,20,29} Additionally, most affected dogs respond to immunosuppressive therapy.^{18,20,29} T cell-mediated PRCA, that accounts for a large portion of primary PRCA in people, has not been documented in dogs.⁶ Human T cell-mediated PRCA has been associated with thymoma, large granular lymphoma, chronic lymphocytic leukemia, other lymphoid malignancies, Epstein-Barr virus infection, and T cell leukemia virus 1 infection.^{9,12} Lymphocytes from patients with these disorders suppress erythropoiesis in colony assays.

Clinical Features

Affected dogs tend to be middle-aged with a greater incidence in females but no known breed predisposition.²¹ Presenting signs include lethargy, collapse, weakness, anorexia, ataxia, weight loss, pica, and seizures. Physical examination findings include mucous membrane pallor, systolic cardiac murmur (due to anemia), tachycardia, and tachypnea.²¹

Laboratory Features

Anemia is severe (median PCV = 10%), reticulocytopenia is profound, and total leukocyte and platelet counts are usually within the reference interval. Spherocytes, microcytes, or nucleated RBCs are observed in less than 20% of affected dogs. Direct Coombs' test results are infrequently positive.²¹ Serum chemistry profiles are generally unremarkable. Bone marrow cellularity is normal or increased with normal numbers of granulocytes and megakaryocytes. Most affected dogs have virtually no erythroid cells in bone marrow aspirates but some have low numbers of rubriblasts and prorubricytes (Fig. 38.1). Increased numbers of plasma cells are present in most affected dogs and small lymphocyte are increased in some dogs.

Differential Diagnosis

Immune-mediated PRCA must be differentiated from other types of nonregenerative IMHA, PRCA associated with parvovirus infection or modified-live parvovirus vaccination, *Ehrlichia canis* infection, myelodysplastic syndromes, or anemias associated with chronic renal failure, and myelofibrosis.^{3,8,13,18,21} Each of these conditions should be considered and appropriate diagnostic tests chosen. The presence of dysplastic features in bone marrow aspiration smears points to myelodysplasia as a cause of the anemia.²³ Dogs not vaccinated or recently vaccinated against parvovirus should be tested by measuring IgG and IgM levels and by testing for parvovirus DNA. Additionally, bone marrow could be evaluated for viral particles. Although chronic ehrlichiosis has not been definitively proven to cause PRCA, testing may be indicated in endemic areas.¹³

Therapy, Course, and Prognosis

Most affected dogs initially require packed RBC transfusions (see Chapter 95). Because response to immunosuppressive therapy is frequently delayed, repeated transfusions may be necessary.

All affected dogs should be treated with immunosuppressive doses of prednisone (1–3 mg/kg PO, q12 hours).¹⁸ Nonsteroidal immunosuppressive agents that may be used in addition to prednisone include azathioprine and cyclophosphamide. The risk of thrombosis appears to be relatively low in PRCA; therefore aspirin or heparin therapy has not been used.²¹ Recombinant erythropoietin therapy has not been extensively used in immune-mediated PRCA.

Response to immunosuppressive therapy is frequently delayed. In one study, median initial response time was 38 days and time required for the PCV to return to the reference interval was 118 days.²¹ Complete hematologic recovery has been reported in 77% of dogs treated with immunosuppressive therapy, and partial recovery was reported in 8%.²¹ In another report, response rate to immunosuppressive therapy was

reported as: complete response 61%, partial response 13% and poor response 26%.¹⁸

Parvovirus Infection- or Vaccination-associated Pure Red Cell Aplasia

Parvovirus infection or vaccination has been incriminated as a possible cause of canine PRCA (see Chapter 19).⁸ Parvovirus B19 infection is a cause of PRCA in humans.³⁰ Parvovirus readily infects hemic precursor cells of dogs and causes acute aplastic anemia.^{3,8} However, severe erythroid and megakaryocytic aplasia has been reported 1–3 weeks after vaccination of dogs with modified live parvovirus vaccine.⁸ A causal relationship has been hypothesized.⁸ In some dogs the aplasia was irreversible and led to death, whereas in other cases the dogs recovered.

Pure Red Cell Aplasia Associated with an Immune Response to Recombinant Human Erythropoietin

Dogs frequently develop PRCA 4–16 weeks after initiation of treatment with recombinant human erythropoietin.¹⁹ The anemia occurs because dogs develop an immune response to the recombinant protein. These antibodies cross-neutralize endogenous canine erythropoietin resulting in profound anemia. The onset of anemia is typically 2–3 months after initiation of therapy and dogs usually recover spontaneously 3–11 weeks after discontinuing treatment.

Congenital Red Cell Aplasia

An inherited PRCA, termed Diamond-Blackfan anemia, has been described in children.¹¹ Congenital PRCA is not established as a diagnosis in dogs. However, one 3-month-old Samoyed was reported to have PRCA and have some features of the human condition.¹⁴ Unlike Diamond-Blackfan syndrome, this dog had a mild thrombocytopenia, had no evidence of illness before 3 months of age, and had no congenital anatomical abnormalities that are seen in Diamond-Blackfan syndrome. Further, vaccination history was not discussed and parvovirus infection was not eliminated as a possible cause of the anemia. Therefore, a congenital origin for this condition remains in question.

PURE RED CELL APLASIA IN CATS

Immune-mediated Pure Red Cell Aplasia

Definition and Incidence

An immune-mediated etiology for PRCA in cats is based on positive direct Coombs' test results in some cats, response to immunosuppressive therapy, and the presence of mature lymphocytosis in bone marrow.^{17,28} In a relatively recent retrospective study of clinical bone marrow reports at a veterinary teaching hospital, PRCA

was diagnosed in 25 of 203 reports (12.6%).²⁴ Only one of these cats tested positive for feline leukemia virus. Another retrospective study conducted at a veterinary teaching hospital identified nine cats with immune-mediated PRCA during a 9 year period.¹⁷ Therefore, reports vary regarding the relative incidence of PRCA in cats.^{6,13}

Clinical Features

Affected cats are usually less than 3 years old.¹⁷ Clinical signs include anorexia, lethargy, and behavioral changes. Physical examination findings include severe pallor of mucous membranes and systolic heart murmur (due to anemia).

Laboratory Features

All affected cats have a severe normocytic normochromic nonregenerative anemia (median hematocrit ~7%), with total leukocyte and platelet counts within or above reference intervals.^{17,28} Bone marrow contains few or no erythroid cells; however, rare rubriblasts and prorubricytes are present in some cats. Dysplastic changes are not a feature of immune-mediated PRCA. The percentage of small lymphocytes is increased in more than 90% of cases and can exceed 50% of all nucleated cells in bone marrow.²⁸ Lymphoid aggregates are frequently seen in bone marrow core biopsy sections.^{22,28} This benign lymphocytosis can be confused with a lymphoid malignancy. Unlike dogs, few cats have plasma cell hyperplasia in bone marrow.²⁴

Differential Diagnosis

Immune-mediated PRCA must be differentiated from other types of nonregenerative IMHA, parvovirus infection, myelodysplastic syndromes, and anemias associated with chronic renal failure, and myelofibrosis.^{26–28} Each of these conditions should be considered and appropriate diagnostic tests chosen. As in dogs, the presence of dysplastic features in bone marrow aspiration smears points to a myelodysplastic syndrome as a cause of the anemia. Cats not vaccinated should be tested for parvovirus infection.

Therapy, Course, and Prognosis

Most affected cats initially require one or more transfusions of packed RBCs (see Chapter 95). Immunosuppressive doses of prednisone (3.5–5.5 mg/kg PO, daily in divided doses) are routinely used for treatment of immune-mediated PRCA.¹⁷ Cyclophosphamide (2.5 mg/kg PO q24 h for 4 days each week) is also frequently used in addition to prednisone.¹⁷ Recombinant human erythropoietin therapy has been used but its benefit is uncertain. Long term use is contraindicated because cats may develop anti-erythropoietin antibodies.

Most cats respond to immunosuppressive therapy. The 60 day survival was reported to be 88% in one study.²⁸ Initial increases in reticulocyte count or hematocrit usually occur within 2–4 weeks after initiation of treatment and hematocrits return to the reference interval within 5 weeks.¹⁷ Despite this rapid response, many cats require prolonged treatment with immunosuppressive drugs to manage this disorder. Some cats relapse when immunosuppressive therapy is withdrawn while other cats do not.

Feline-Leukemia Virus-induced Pure Red Cell Aplasia

Definition and Pathogenesis

Feline leukemia is currently classified into subgroups A, B, and C.⁷ Subgroup C arises de novo from subgroup A through mutation or recombination with endogenous FeLV sequences. Studies with cloned FeLV have shown that FeLV subgroup C uniquely produces PRCA and results in a fatal anemia.⁷ The genetic determinant of PRCA codes for the N-terminal 241 amino acids of an envelope gp70.^{7,15} Anemogenesis may be mediated by an interaction of viral gp70 with a receptor on erythroid progenitor cells. However, the anemogenic activity of FeLV subgroup C is not dependent on unique tropism. Both FeLV subgroup C and FeLV subgroup A infect all three cell lineages in feline bone marrow; however, only subgroup C selectively eliminates erythroid precursor cells.⁷ Erythroid burst-forming units (BFU-E) appear to be uniquely sensitive to FeLV subgroup C suppression. Erythroid colony-forming units (CFU-E) disappear 2–3 weeks before the onset of anemia in cats experimentally infected with FeLV subgroup C/Sarma while BFU-E and granulocyte-macrophage colony-forming units persist.¹ These data indicate that the gp70 of FeLV subgroup C/Sarma interferes with differentiation of BFU-E to CFU-E. The mechanism by which BFU-E fails to differentiate into CFU-E is incompletely understood. BFU-E fails to respond to hematopoietic growth factors and has an abnormal complement sensitivity.²

Clinical Features

Although FeLV-induced PRCA has been repeatedly induced experimentally, the clinical importance of the virus as a cause of PRCA is uncertain. Under experimental conditions, susceptibility to FeLV is age-related. Neonatal kittens are most sensitive with older kittens developing significant natural resistance by 4 weeks of age. Kittens inoculated with FeLV-KT became anemic in 3–8 weeks, whereas immunosuppressed cats inoculated with FeLV subgroup C became anemic after 9–45 weeks.^{2,4}

Laboratory Features

Cats experimentally infected with FeLV subgroup C have a profound anemia (hematocrit < 10%), reticulocytopenia, and an absence of identifiable erythroid

precursor cells in bone marrow.¹⁶ Granulocyte and megakaryocyte precursors are usually normal. Extramedullary hematopoiesis is absent in the disorder. Some cats develop osteosclerosis or myelofibrosis with associated pancytopenia (see Chapter 18).

Course and Prognosis

FeLV-associated PRCA does not respond to immunosuppressive therapy or other known therapies. The anemia is unrelenting and disease progression is rapid despite therapeutic efforts.¹⁶ Blood transfusions offer short term relief from the anemia. At present the prognosis is grave.

Pure Red Cell Aplasia Associated with an Immune Response to Recombinant Human Erythropoietin

As in dogs, cats can develop PRCA after treatment with recombinant human erythropoietin. Unlike dogs, cats can also develop PRCA after treatment with recombinant feline erythropoietin. The anemia results from an immune response to the recombinant protein that cross-neutralizes endogenous feline erythropoietin and results in profound anemia. Cats usually recover spontaneously after treatment is discontinued.

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Aplastic Anemia

DOUGLAS J. WEISS

Pathogenesis

Diagnosis

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Mycotoxin Ingestion

Acronyms and Abbreviations

FeLV; feline leukemia virus; RBC, red blood cell.

Aplastic anemia (also termed aplastic pancytopenia) is a clinical syndrome characterized by bicytopenia or pancytopenia in the blood and replacement of bone marrow by adipose tissue (Fig. 39.1).^{4,22} Hematopoietic cells are nearly absent and the low number of cells that remain usually consist of lymphocytes and plasma cells. Excluded from this are pancytopenias associated with myelophthisic disorders, such as secondary myelofibrosis, and leukemias, hypoplastic myelodysplastic syndromes, pure red cell aplasia, hemophagocytic syndrome, myelonecrosis, and inflammatory disorders of bone marrow.⁴

Aplastic anemia can occur in acute and chronic forms. If the bone marrow suppression occurs acutely, as is seen in some types of drug- or virus-induced aplasia, neutropenia typically develops within 1 week followed by thrombocytopenia at 9–14 days.²⁸ Anemia does not develop for an extended period of time, because of the long lifespan of red blood cells (RBCs). Therefore, acute aplastic anemia is typically characterized by a neutropenia and thrombocytopenia. Acute aplastic anemia is most frequently associated with destruction of rapidly dividing progenitor or proliferative cells in bone marrow. In chronic aplastic anemia, the hematologic dyscrasia develops more slowly and is characterized by moderate to severe normocytic normochromic nonregenerative anemia, neutropenia, and thrombocytopenia.^{22,14} Chronic aplastic anemia is most frequently associated with stem cell destruction.²²

PATHOGENESIS

A large variety of etiologic agents have been incriminated in aplastic anemia. These include infectious agents, chemical/physical agents, drugs, toxins, and immune-mediated causes. The end result of each of these insults is decreased blood cell formation.^{22,28} Although several mechanisms could lead to decreased hematopoiesis, direct toxic injury to stem cells or progenitor cells appears to be the primary mechanism involved in clinical aplastic anemia.^{17,18} Other possible mechanisms include an altered stromal microenvironment resulting in failure to hematopoiesis, impaired production of hematopoietic growth factors, genetic mutations that decrease the proliferative capacity of stem cells, and immune-mediated suppression of hematopoiesis.²² There is little experimental evidence to support defects in hematopoietic growth factors, the stromal microenvironment, or genetic mutations. T cell immunosuppression has been incriminated in the pathogenesis of idiopathic aplastic anemia in humans.³² The role of autoreactive T cells in human aplastic anemia is supported by coculture studies in which marrow lymphocytes from affected patients suppressed hematopoietic colony formation of normal marrow.¹⁹ Cytokines with inhibitory effects on hematopoiesis, including interferon-gamma, interleukin-2, and tumor necrosis factor-alpha, are increased in the marrow of patients with idiopathic aplastic anemia.¹⁶ Therefore,

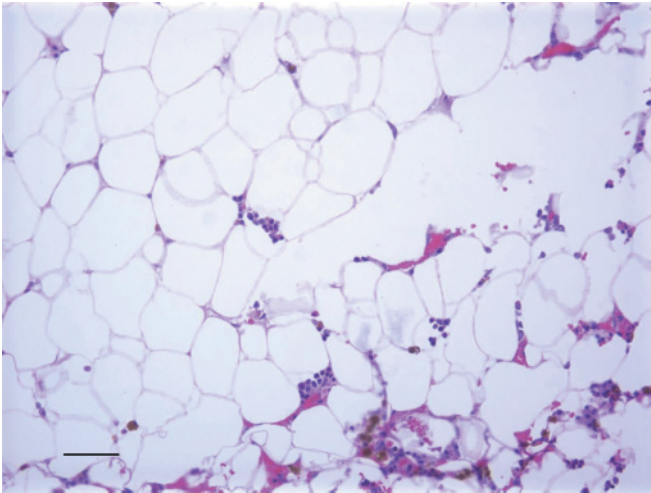


FIGURE 39.1 Bone marrow core biopsy section from a dog with idiopathic aplastic anemia. Cells present are mostly small lymphocytes and plasma cells. Hematoxylin & Eosin stain; bar = 200 μ m.

immune inhibition in human idiopathic aplastic anemia is mediated in part by inhibitory cytokines released by cytotoxic T cells.¹⁶ The autoreactive T cell etiology for idiopathic aplastic anemia is further supported by clinical trials in which anti-lymphocyte globulin has been used as an effective therapy for aplastic anemia.²² Additionally, certain genetic mutations appear to predispose people to aplastic anemia.²⁴

DIAGNOSIS

Inclusion criteria for a diagnosis of aplastic anemia in humans are well defined. They include: hemoglobin concentration <10 g/dL, reticulocytopenia, platelet count $<50 \times 10^3/\mu\text{L}$, a neutrophil count $<1.5 \times 10^3/\mu\text{L}$, and hypocellular bone marrow aspirates and core biopsies as well as a battery of ancillary diagnostic tests including cytogenetic analysis of the peripheral blood and bone marrow, fetal hemoglobin percentage in children, flow cytometry for phosphatidylinositol glycan-anchored proteins, liver function tests, vitamin B₁₂ and folate concentrations, viral studies, testing for immune-mediated diseases, and diagnostic imaging.¹⁴ Diagnostic recommendations for aplastic anemia in veterinary patients are not well defined and currently minimally include evaluation of a complete blood count, bone marrow aspiration smear, and core biopsy specimen.^{4,28} Fat content of bone marrow depends on location and depth of core biopsy. The bone marrow collection sites should be carefully chosen as fatty marrow samples can be obtained in healthy animals if an improper collection site is selected.³⁰ Ancillary diagnostic tests should be chosen based on the species and case history. Animals should be evaluated for drug, chemical, and toxin exposure, exposure to specific infectious agents and the presence of associated diseases such as chronic renal failure.⁴

Infectious agents in the dog include *Ehrlichia canis* and parvovirus infection, in the cat include feline leukemia virus and parvovirus infection, and in horses include equine infectious anemia infection.^{4,26,28}

MODELS OF APLASTIC ANEMIA

A variety of in vivo and ex vivo animal models of aplastic anemia have been described.⁵ A mouse model has been used to study benzene-induced aplastic anemia. Use of this model has enabled investigators to identify a benzene-induced defect in the capacity of bone marrow stromal cells to process cytokines. A mouse model of busulfan-induced aplastic anemia has been extensively studied.⁵ Busulfan treatment results in hematopoietic stem cell depletion with pancytopenia and bone marrow aplasia developing up to a year after treatment. Other studies suggest the presence of a qualitative defect in hematopoietic stem cells or hematopoietic stromal cells. Radiation injury has also been studied in several species.⁶ Exposure of mice to 4Gy ionizing radiation resulted in significant reduction in hematopoietic stem cells.

Immune-mediated aplastic anemia has been reproduced in mice by sublethally irradiating them and injecting them with heterologous lymphocytes.⁵ Total body irradiation with 6Gy and injection of 10 million lymph node cells produced a fatal pancytopenia within 2–3 weeks. Aplasia was shown to be dependent on the presence of T cells in the infused lymphocyte population.

APLASTIC ANEMIA IN DOGS

Aplastic anemia was identified in 2.4% of clinical bone marrow specimens evaluated at a veterinary teaching hospital.²⁸ Of these, the majority (1.7%) were idiopathic.⁴ Other causes of aplastic anemia included infectious causes, drugs, toxins, and radiation.

Infectious Causes

Infectious agents that cause aplastic anemia include parvovirus and *Ehrlichia canis* (see Chapters 19 and 31). Puppies with parvovirus infection develop erythroid and myeloid hypoplasia.³ This is not a true aplastic anemia because the hypoplasia is accompanied by degenerative changes including myelonecrosis, edema, and hemorrhage (see Chapter 17).³ These could be caused by the cytotoxic effects of the virus or more likely the effects of secondary bacterial sepsis or endotoxemia on the marrow microvasculature. Parvovirus invades rapidly dividing myeloid and erythroid progenitor cells. Hematopoietic stem cells are not infected. Therefore, bone marrow rapidly regenerates if puppies survive the acute stage of the disease. Dogs with chronic *Ehrlichia canis* infections develop hypoplastic bone marrows.¹⁰ Rarely aplastic anemia may occur.

Drug-associated Causes

Drugs that induce hematologic dyscrasias are discussed in Chapter 16. In this chapter, some general comments about drug-induced aplastic anemia are presented. The mechanism by which the drug induces suppression of hematopoiesis is important to clinical finding and outcome. Drugs that target progenitor or proliferative cells in marrow, such as anti-neoplastic drugs and sulfadiazine, result in acute aplastic anemia.²⁹ In some cases these drugs are directly cytotoxic to rapidly proliferating hematopoietic cells, whereas other drugs act indirectly by inducing immune-mediated hematopoietic cell destruction. Affected dogs are typically neutropenic and thrombocytopenic but not anemic, and frequently undergo prompt hematologic recovery when the drug is withdrawn.²⁹ Drugs that target stem cells, such as estradiol and phenylbutazone, frequently cause chronic aplastic anemia that is characterized by pancytopenia.²⁹ Recovery is uncertain and when it occurs it may be delayed for months.

Canine bone marrow is uniquely susceptible to estrogen-induced suppression (see Chapter 16).²⁹ In dogs, a single large dose of estradiol, repeated therapeutic doses, and elevated endogenous estrogen levels associated with cystic ovarian follicles, ovarian granulosa cell tumors, or Sertoli cell tumors, result in hematologic dyscrasias.²³ Dogs with endogenous estrogen toxicity typically present with pancytopenia but some are bicytopenic.²³ Bone marrow is usually characterized by panhypoplasia; however, in the early stage, some dogs have granulocyte hyperplasia. Administration of a single large dose of estradiol to dogs results in a consistent transient aplastic anemia.²² Individual dogs appear to be uniquely sensitive to the effects of estrogen and develop severe aplastic anemia. This appears to be the result of hematopoietic stem cell destruction. The prognosis for these dogs is poor but recovery after weeks to months of supportive care has been documented.²⁹

Idiopathic Aplastic Anemia

Idiopathic aplastic anemia was observed in 1.7% of clinical bone marrow specimens examined at a veterinary teaching hospital.⁴ Affected dogs tend to be young adult with no sex or breed predisposition. The history usually indicates a chronic onset of illness with nonspecific signs including lethargy, anorexia, and exercise intolerance. Fever, petechiae, and epistaxis may also be observed. The anemia varies from mild to severe and is normocytic and normochromic. Neutropenia is usually severe with leukocytes consisting mostly of small lymphocytes. Thrombocytopenia varies from moderate to severe. Bone marrow aspirates and core biopsy specimens are markedly hypocellular. In some dogs, small lymphocytes or plasma cells are increased in number.

Treatment of canine idiopathic aplastic anemia is not well defined and is mostly supportive. Whole blood, packed RBC, or platelet transfusions are frequently

needed. Broad-spectrum antibiotics are indicated to prevent opportunistic infections. Because of the clear connection with the human disease, a primary immune-mediated destruction of hematopoietic precursor cells has been suspected in canine idiopathic aplastic anemia. However, the author has found that administration of immunosuppressive doses of prednisone is ineffective as a treatment. This is not surprising because idiopathic aplastic anemia in humans only responds to intensive immunosuppressive therapy that includes anti-thymocyte globulin and steroidal and non-steroidal immunosuppressive drugs.²²

Outcome of idiopathic aplastic anemia is variable. In one study of seven affected dogs, two dogs had complete hematologic recovery with symptomatic therapy and were alive more than 5 years after diagnosis.⁴ Therefore, although the prognosis is guarded to poor, some have complete clinical recoveries.

APLASTIC ANEMIA IN CATS

Aplastic anemia has been observed in 10% of feline clinical bone marrow specimens at a veterinary teaching hospital.³¹ Conditions associated with aplastic anemia include chronic renal disease, feline leukemia virus (FeLV) infection, prolonged anorexia/starvation, and adverse drug reactions including griseofulvin, chemotherapeutic agents, propylthiouracil, and methamizole.²⁷ Chronic renal disease is typically associated with nonregenerative anemia, and aplastic anemia is an infrequent finding. Some cats with aplastic anemia associated with chronic renal disease have serous atrophy of fat in bone marrow (see Chapter 18).²⁷ Serous atrophy of fat is usually associated with starvation. Starvation has been implicated in the pathogenesis of the marrow failure in people and other species.

FeLV infection has been associated with a variety of bone marrow disorders including aplastic anemia.^{7,27} Cats usually have a severe normocytic normochromic anemia (hematocrit <10%), severe leukopenia, and thrombocytopenia. Affected cats frequently have secondary bacterial infections as a result of the neutropenia. Bone marrow is aplastic; however, the pathogenesis of the aplasia is unclear. Survival time for FeLV-associated aplastic anemia is usually short.

In one study of aplastic anemia in cats, 30% were classified as idiopathic.²⁷ However, most cats had associated disease conditions. The most frequent diseases included oral and gastrointestinal ulcers. Many affected cats had a history of prolonged partial or complete anorexia with associated cachexia.²⁷ One case had serous atrophy of fat and gelatinous transformation in bone marrow. Therefore, starvation may have played a role in the development of the aplasia in these cats.

Although not recognized as a clinical disorder, cat bone marrow appears to be uniquely sensitive to mycotoxins. Mycotoxin exposure results from ingestion of grain that becomes damp during storage permitting growth of fungi. In a search for an experimental model

for T-2 mycotoxicosis, sesquiterpene T-2 mycotoxin was administered to rats, mice, guinea pigs, rabbits, dogs, pigs, sheep, poultry, cattle, horses, and cats.¹³ Only cats developed signs of hematotoxicity. This was characterized by pancytopenia and bone marrow was characterized by hypocellularity, hemorrhage, inflammation, and multinucleated giant cells. Although this is consistent with acute toxic injury rather than aplastic anemia, the bone marrow may become aplastic at a later stage of the disease.

APLASTIC ANEMIA IN HORSES

Several reports of equine aplastic anemia have been published. These reports appear to present distinct types of bone marrow aplasia including idiopathic, drug-associated, possible toxin-associated, genetic, and hypoplastic myelodysplastic syndrome. A case of idiopathic aplastic anemia was reported in a 2-year-old Quarter Horse.² This horse had severe pancytopenia and bone marrow aplasia typical of aplastic anemia and had no history of exposure to drugs, toxins, or infectious agents. The horse died from hemorrhage as a result of the bone marrow biopsy procedure.

Several suspected drug-induced aplastic anemia cases have been reported. Phenylbutazone has been incriminated as a cause of aplastic anemia.⁸ A 4-year-old Quarter Horse developed aplastic anemia after treatment with penicillin, trimethoprim-sulfadiazine, oxytetracycline, phenylbutazone, dipyrone, flunixin meglumine, and isoxsuprine.¹¹ This horse was also direct Coombs' test positive and had a transient increase in the blood of large immature basophilic cells resembling lymphoblasts. Therefore, it is difficult to incriminate any one drug as the cause of the aplasia, but penicillin and sulfadiazine are known to induce immune-mediated hematologic disorders. The horse had prompt and complete clinical and hematologic recovery after discontinuation of treatment.

Aplastic anemia in a 9-week-old Clydesdale foal was associated with biliary hyperplasia and portal fibrosis.¹⁵ Because of this combination of findings, an in utero or neonatal toxicity was suspected. A panhypoplasia of bone marrow has been reported in eight Standardbred horses sired by the same stallion.⁹

Finally a severe nonregenerative anemia and leukopenia that initially had an aplastic bone marrow was reported in a 17-year-old Thoroughbred gelding.²⁵ Unusual features of this case were marked anisocytosis in the blood, normal numbers of megakaryocytes in bone marrow, and an increase in blast cells in bone marrow. Re-evaluation of bone marrow several months later revealed a hypercellular marrow with an increase in lymphocytes and blasts cells. This is suggestive of hypoplastic myelodysplastic syndrome (see Chapter 66).

Based on these sparse and varied reports, aplastic anemia appears to occur infrequently in horses. Drug-induced causes should be considered in all horses. In

older horses leukemia and myelodysplastic syndromes may induce pancytopenia.

APLASTIC ANEMIA IN RUMINANTS

Drug-Associated Causes

A case of aplastic anemia in a 14-day-old Holstein heifer was associated with prior administration of sulfamethazine, trimethoprim, and sulfadoxine for treatment of bloody diarrhea.¹ Detection of aplastic bone marrow 8 days after initiation of sulfa therapy fits with a drug-induced aplastic anemia.

Bracken Fern Ingestion

Bracken fern (*Pteridium aquilinum*) is typically eaten by ruminants when pastures are overgrazed. After repeatedly ingesting bracken fern, cattle, horses, sheep, and pigs develop aplastic anemia.¹⁹ The major toxin is a thiaminase called ptaquiloside.³¹ Thiamine depletion in cattle is thought to be involved in the pathogenesis of aplastic anemia. Other species are more likely to develop neurological signs related to polioencephalomalacia.³¹ Development of urinary bladder neoplasia (i.e. chronic enzootic haematuria) is also linked to prolonged ingestion of bracken fern.¹⁹

Trichloroethylene-extracted Soybean Oil Ingestion

Many cases of fatal aplastic anemia and renal injury in cattle in the 1950s were traced to feeding of trichloroethylene-extracted soybean oil meal.^{12,21} Although trichloroethylene is no longer used to extract soybean oil, it and related compounds are widely used as solvents. The toxic factor was identified as S-(1-2-dichlorovinyl)-l-cysteine. Administration of a single dose of this compound intravenously at 4 mg/kg or 0.4 mg/kg per day intravenously for 10 days reproduces the aplastic anemia and renal injury.

Mycotoxin Ingestion

A variety of mycotoxins produce aplastic anemia in ruminants. Mycotoxin exposure occurs with ingestion of grain that is contaminated with fungi as a result of becoming damp during storage. Aplastic anemia has been documented in cattle and sheep ingesting feed contaminated with *Stachybotrys* spp.²⁰ Many other mycotoxins, including T-2 mycotoxin, trichothecene mycotoxin, trichothecene vomitoxin, diacetoxyscirpenol, ochratoxin, and zearalenone, appear to be directly cytotoxic to a variety of cells including hematopoietic precursor cells and lymphocytes.⁶ As a result, many mycotoxins cause bone marrow hypoplasia/aplasia and lymphocyte depletion.⁶ Initial changes in bone marrow consist of individual cell necrosis or apoptosis; however, by the time affected animals are evaluated

clinically the bone marrow frequently appears aplastic.⁶ Depletion of primary and secondary lymphoid tissue may also be seen.

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A microscopic image of a blood smear showing various types of leukocytes. The background is a light blue color. Several large, irregularly shaped cells with dark, multi-lobed nuclei are visible, characteristic of neutrophils. There are also smaller, more rounded cells with dark nuclei, likely lymphocytes. The overall appearance is that of a typical white blood cell count slide.

SECTION IV

Leukocytes

Erik Teske

Neutrophil Structure and Biochemistry

MARY B. NABITY and SHASHI KUMAR RAMAIAH

Neutrophil Ultrastructure
 Neutrophil Granule Types
 Neutrophil Granule Structure
 Granule Function
 Granule Contents
 Species Differences

Antimicrobial Activity of Neutrophils (see Chapter 41)
 Neutrophil Surface and Associated Receptors (see Chapter 41)

Acronyms and Abbreviations

BMAP, bovine myeloid antimicrobial peptide; BPI, bactericidal/permeability-increasing protein; eCATH, equine cathelicidin; e-NAP, equine neutrophil antimicrobial peptide; FMLP, formyl-methionyl-leucyl-phenylalanine; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; hCAP, human cathelicidin protein; K9CATH, canine cathelicidin; LAMP, lysosome-associated membrane protein; MPO, myeloperoxidase; NADPH, nicotinamide adenine dinucleotide phosphate; Nramp1, natural resistance-associated macrophage protein 1; PMAP, porcine myeloid antimicrobial peptide; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor.

Neutrophils provide the first line of defense against invading microorganisms, tissue trauma, or any inciting inflammatory signal.¹ The capacity of neutrophils to systematically engulf and fight bacteria depends on the presence of numerous membrane receptors and subsequent cell signaling along with specific cytoplasmic constituents within granules. The goal of this chapter is to provide a detailed overview of the constituents of cytoplasmic granules and associated membrane receptors and their functions. An attempt will also be made to provide comparative species differences whenever possible. For additional information on neutrophils, readers are referred to Chapters 4, 7, 41–43, and Section IX.

NEUTROPHIL ULTRASTRUCTURE

Mature, segmented neutrophils are approximately 10–12 μm in diameter.⁵ Scanning electron microscopy shows that their surface contains numerous small pseudopodia (Fig. 40.1). This increases the surface area of the neutrophil, which is crucial to phagocytic function.²¹ Mature neutrophils have a lobulated nucleus composed mostly of heterochromatin with a thin strand of euchromatin in the center. A nucleolus is only rarely observed, and few nuclear pores are present.⁴ With the exception

of numerous granules, neutrophil cytoplasm contains few organelles (mitochondria, Golgi apparatus, endoplasmic reticulum) and a variable number of glycogen particles. A thin rim at the periphery of neutrophils typically contains a small area free of organelles and inclusions but with numerous cytoskeletal proteins.⁴

NEUTROPHIL GRANULE TYPES

Neutrophils contain a variety of granules that contribute to the first-line host defense against bacteria, fungi, protozoa, and some viruses. These granules contain many proteins including antimicrobial proteins, proteases, and components of the respiratory burst, as well as receptors for endothelial adhesion molecules, extracellular matrix proteins, bacterial products, and soluble mediators of inflammation.¹² These proteins must be properly packaged and exocytosed in order to prevent widespread damage to host tissue.²⁴ The granule types and contents have been best characterized in humans; therefore this section focuses predominantly on what is known regarding human neutrophils with any identified species differences being noted.

In humans, there are three main granule types present in mature neutrophils: primary (azurophil or myeloperoxidase-positive), secondary (specific), and tertiary

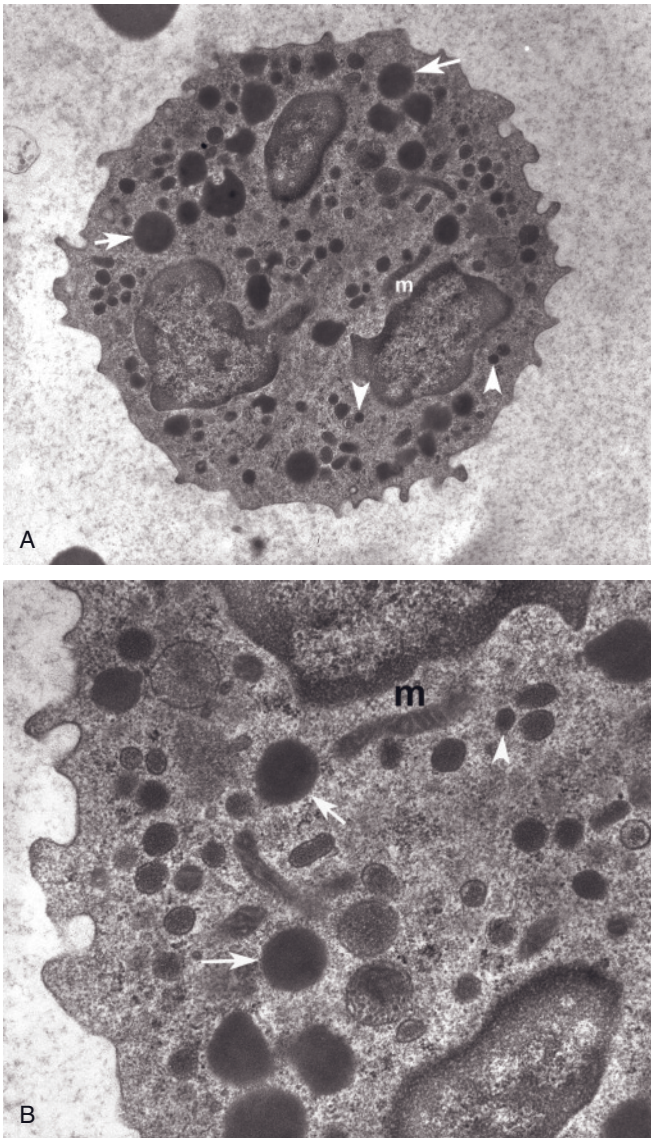


FIGURE 40.1 (A, B) Transmission electron micrographs of a segmented canine neutrophil. The neutrophil contains numerous variably-sized round to oval primary (arrows) and secondary (arrowheads) secretory granules. Several nuclear lobes are visible with marginated heterochromatin and central euchromatin. Few organelles, including rare mitochondria (m), are present in a cytosol replete with electron-dense glycogen granules. Stained with uranyl acetate and lead citrate. (A) $\times 8400$ and (B) $\times 20,000$. (Courtesy of Dr. Craig Thompson, Purdue University, College of Veterinary Medicine.)

(gelatinase) granules. Secondary and tertiary granules are both peroxidase-negative. Other similar structures present are secretory vesicles and multilaminar and multivesicular bodies. Secretory vesicles, which contain plasma proteins, are likely formed by endocytosis;⁶ therefore they are classified as endocytic vesicles rather than true granules. Multilaminar and multivesicular bodies may be precursors to “housekeeping” lysosomes. Typical dense, mature lysosomes found in most other cells have not been identified in neutrophils.⁸ Until fairly recently, primary granules were thought to be primary

lysosomes. Like lysosomes, their membrane contains CD63 (lysosomal membrane-associated protein-3; LAMP-3) and CD68, but they do not have LAMP-1 or LAMP-2.¹¹ They, along with the other granules, may therefore be more accurately referred to as regulated secretory granules or lysosome-related organelles.^{8,11}

Granules are, for the most part, formed sequentially during neutrophil maturation (Fig. 40.2). Primary granules are the first to be formed in the promyelocyte stage, while secondary granules are formed during the myelocyte and metamyelocyte stages.² Tertiary granules and secretory vesicles both appear to be formed in the band and segmented neutrophil stages.⁶ Mature neutrophils therefore contain a mixture of granules and vesicles.

NEUTROPHIL GRANULE STRUCTURE

Neutrophil granules have a phospholipid bilayer membrane surrounding an intragranular matrix that contains proteins for either exocytosis into the environment or fusion with a phagosome. In most species, primary granules are the largest and most dense of the granules based on subcellular fractionation in a density gradient.⁴ Primary granules can have great heterogeneity in size and shape.⁶ Secondary granules are smaller, less dense, and more numerous than primary granules (Fig. 40.1).⁴ They can be round, oval, elongated, or dumbbell shape.⁶ Tertiary granules are slightly smaller than secondary granules. Secretory vesicles range from 100 to 200 nm in size and are widely distributed within the cytosol similarly to granules.²⁶ They do not have defining ultrastructural characteristics and have been identified on electron microscopy by double-labeling with anti-albumin and either anti-CD11b/CD18 (Mac 1) or anti-cytochrome b_{558} antibodies.²⁶

The bulk of evidence supports the hypothesis that granule proteins are predominantly segregated into their respective granule by a “targeted-by-timing” expression of proteins rather than an active sorting mechanism.^{12,15} In the targeted-by-timing theory, the proteins are produced by the endoplasmic reticulum in response to regulated expression of transcription factors present at specific stages of neutrophil development (e.g. PU.1, AML-1, C/EBP ϵ).⁷ Therefore, whichever proteins are being synthesized at the time a particular granule is produced will be incorporated into that granule.^{10,12} For example, primary granules are formed during the promyelocyte stage, when myeloperoxidase is expressed.⁶ However, not all protein differences among the granules can be explained by timing of their synthesis, because defensins are produced in both early and later stages, but are not incorporated into secondary and tertiary granules.^{3,12,15}

GRANULE FUNCTION

The separation of proteins into different granules/compartments serves two major purposes. First, it separates

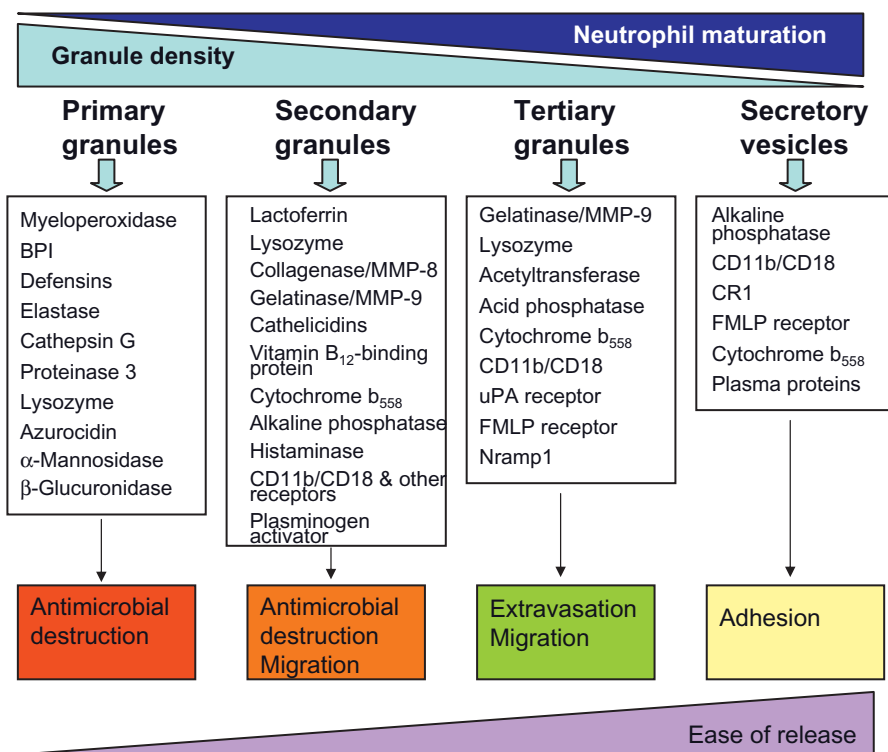


FIGURE 40.2 Neutrophil granules, contents, and their functions. Neutrophils contain primary, secondary, and tertiary granules and secretory vesicles that contain many proteins involved in neutrophil-mediated attack on microorganisms. The granules and their contents are sequentially formed during neutrophil maturation. The above figure contains a partial listing of granule contents. Although the granules have many overlapping functions, a simplified summary of the primary role of each granule is shown at the bottom of the figure. (Adapted from Gullberg U, et al. Processing and targeting of granule proteins in human neutrophils. *J Immunol Methods* 1999;232:201–210, with permission.)

proteins that cannot coexist together without the risk of activation of enzymes, protein degradation or cellular damage. Second, it allows for differential exocytosis of proteins based on varying degrees of neutrophil stimulation. This fulfills the need to have proteins available for use at different times and in different contexts to perform the multiple functions of the cell.

Granule release is dependent on receptor-mediated neutrophil stimulation by a variety of molecules. One of the frequently used activators studied *in vitro* is formyl-methionyl-leucyl-phenylalanine (FMLP), a bacterial peptide. This peptide illustrates the marked variation across species seen in neutrophil activation responses. For instance, neutrophils of humans, non-human primates, rabbits, guinea pigs, and mice are all activated by FMLP. However, neutrophils of cattle, pigs, dogs, and cats have no FMLP response presumably due to a lack of FMLP receptors.²⁸

When granules are released, their contents can either be released into phagosomes (e.g. primary granules) or they can be released into the extracellular environment (e.g. secondary and tertiary granules).¹⁹ During exocytosis, the granule or vesicle membrane becomes incorporated into the phagosome or neutrophil membrane, respectively. This concept of membrane fusion is crucial, as the granule/vesicle membranes contain many differ-

ent receptors necessary for neutrophil extravasation, chemotaxis, migration, and immunomodulation.

There is significant overlap in protein content and function of these granules; however, it is useful to consider the general classes of enzymes and proteins present in each granule in order to highlight their primary function (Fig. 40.2). In general, their ease of exocytosis is inversely related to the order of the formation of the granules.^{6,20} Secretory vesicles are the first to be released upon neutrophil stimulation, and they are rapidly and completely mobilized early during neutrophil-endothelial interactions. At the same time, L-selectin is shed from the neutrophil surface.^{12,26} Secretory vesicle membranes contain adhesion proteins (e.g. CD11b/CD18), chemoattractant receptors, and complement receptors that are incorporated into the neutrophil cell membrane after exocytosis.²⁶ These receptors permit the neutrophil to establish firm adhesion with the endothelium, and aid in subsequent diapedesis and chemotaxis.⁶ Tertiary granules are the next most readily released. They contain matrix-degrading enzymes (e.g. gelatinase) and membrane receptors that aid in neutrophil extravasation and migration.¹² Next to be mobilized are secondary granules, which contain many antimicrobial substances but also contain some matrix-degrading metalloproteinases. Secondary

granules, along with the primary granules, participate mainly in microbial destruction. The primary granules require the greatest stimulus in order to be released (Fig. 40.2).⁶

Granule Contents

Despite a large degree of overlap in the protein content among granules, there are several important proteins characteristic of certain granules. By definition, primary granules contain myeloperoxidase (MPO), a potent microbicidal hemoprotein involved in the oxygen-dependent destruction of microorganisms.^{6,20} Other typical proteins found within primary granules include alpha-defensins, bactericidal/permeability-increasing protein (BPI) and the serine proteases such as elastase, cathepsin G, and proteinase 3. Recently, granzyme B has been identified in primary granules of human neutrophils.²⁹

Defensins are powerful pore-forming antimicrobial peptides that are produced during the late promyelocyte and myelocyte stage. Primary granules can, therefore be divided into two types: early-appearing, defensin-poor primary granules and late-appearing, defensin-rich primary granules.¹² Bactericidal/permeability-increasing protein binds lipopolysaccharide of Gram-negative bacteria, inducing growth inhibition and membrane damage. It also mediates phagocytosis of bacteria.¹² The serine proteases can degrade extracellular matrix, aid in microbial death, and regulate the immune response.¹⁶ Many of the proteins present in the primary granules are synthesized as proforms but the propeptides are removed during granule maturation, and they are therefore stored in their active conformation.¹²

Secondary granules also contain large amounts of antimicrobial substances, particularly lactoferrin, which is their main defining protein. Other antimicrobial proteins present in secondary granules include hCAP-18 (a cathelicidin), alkaline phosphatase, and lysozyme.¹² Secondary granules also contain several matrix-degrading enzymes such as collagenase and a small amount of gelatinase. The membrane of secondary granules is an important source of cytochrome b₅₅₈, a component of NADPH oxidase.⁶ Enzymes in secondary granules are stored as inactive latent proforms and are activated upon exocytosis.

Tertiary granules have a large amount of gelatinase and only a small amount of lactoferrin. They also contain many other enzymes, including lysozyme. Tertiary granule membranes contain many important receptors and proteins, including natural resistance-associated macrophage protein 1 (Nrap1), which may help deprive microorganisms of divalent cations.¹²

Secretory vesicle membranes contain many receptors important in extravasation and phagocytosis (CD11b/CD18, CR1, FMLP-receptor, CD14) in addition to alkaline phosphatase. Their matrix contains plasma proteins, including albumin, while antimicrobial enzymes are absent.²⁶ A partial listing of granule contents is given in Fig. 40.2.

Neutrophil granules also contain anti-inflammatory proteins, and a recent study found the acute phase protein alpha₁-acid glycoprotein in bovine neutrophil granules.²³ Alpha₁-acid glycoprotein modulates the expression of anti-inflammatory cytokines by macrophages and decreases neutrophil chemotaxis, among other anti-inflammatory functions. This may provide a feedback mechanism whereby neutrophils can modulate their responses locally, limiting host tissue damage.²³

Species Differences

There is a large degree of interspecies variability in the quantitative presence and activity of each granule element, and species-specific distribution of granule constituents may affect the pathogenicity of microorganisms in different species.^{21,26,27} However, species-specific distribution of granule constituents is not well defined. Only a limited number of enzymes and proteins have been evaluated in species other than humans. To complicate comparison among those species that have been studied, most studies of neutrophil physiology and function use cells from a single species and use different collection, isolation, and processing techniques.²⁸

Despite the relative lack of information regarding species differences in neutrophil granules, some conclusions can be made. First, it appears that most species studied contain primary and secondary granules, and the most frequent enzymes evaluated within these granules appear to be similarly distributed across species. Second, many species (cattle, sheep, goats, dogs, cats, rabbits, rats, guinea pigs, and horses) appear to have a third granule type that may or may not correlate in function with tertiary granules in humans.^{4,13,27}

The third neutrophil granule in cattle has been the most characterized of the above species, and the third granule of sheep and goats appears similar. In cattle, these granules are large, dense, and peroxidase-negative and are referred to as "large" or "dense" granules.^{14,30} They do not appear to be equivalent to the tertiary granules of humans. Ultrastructurally, they are the largest (0.4–0.5 μm) and most numerous granules in mature bovine neutrophils, and they contain pale homogeneous matrix material.¹⁴ They are readily mobilized and are responsible for the majority of antimicrobial protein activity in bovine neutrophils, containing lactoferrin, batenecins (members of the cathelicidin family), and beta-defensins.³⁰ They are the only neutrophil granule known to contain defensins in the bovine, in contrast to humans, where defensins are localized to primary granules.³⁰

Regarding enzyme content, there is marked variation in neutrophil enzyme activity among species, which could be explained by an actual difference in neutrophil content of the enzyme, differences in the biochemistry and detection of the enzyme by the assay employed, or the presence of the enzyme in its inactive form.²⁵ Most information is based on studies that evaluate activity within the entire neutrophil rather than activity within individual granules.

In these studies, beta-glucuronidase and MPO were present to some degree in neutrophils of all species evaluated except chickens and geese. However, MPO activity was typically lower in animals compared to humans except for dogs and non-human primates.^{25,28} A relatively recent study found that chicken heterophils contain MPO activity, contrary to the earlier studies showing a lack of MPO activity in avian heterophils.¹⁷ Neutrophil lysozyme was deficient in monkeys, cattle, goats, sheep, cats, and hamsters, and only a small amount of activity was detected in horses, dogs, rats, and guinea pigs.^{25,28} Alkaline phosphatase was undetectable in rhesus monkeys, cats, some mouse strains, chickens and geese, and was low in dogs.²⁵ A small degree of elastase activity was present in horse and pig neutrophils versus a large amount present in the rabbit.²⁸ Bovine neutrophils contained high levels of arylsulfatase, and both bovine and porcine neutrophils contained low levels of beta-glucuronidase.²⁸

Alpha-defensins have been identified in neutrophils of humans, rabbits, guinea pigs, and hamsters, but not in mice, cattle, pigs, and birds. Beta-defensins have been identified in neutrophil dense granules of cattle and in avian heterophils, but not in neutrophils of sheep.¹⁸ Theta-defensins are a newly described class of defensins that are present in granulocytes of rhesus macaques and other Old World monkeys and orangutans but not in great apes, humans, and New World monkeys.¹⁸ Horse neutrophils contain only trace amounts of defensins but they do contain significant amounts of equinins (e-NAP1 and e-NAP2). These novel proteinase inhibitors have antibacterial and antiviral activities and are structurally different from defensins.^{9,22} Cathelicidins are present in neutrophils of dogs (K9CATH), horses (eCATH), cattle (bactenecins, BMAPs), sheep, pigs (protegrins, prophenins, PMAPs), rabbits, guinea pigs, rats, mice, and birds.¹⁸

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Neutrophil Distribution and Function

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Adherence

Selectins

Integrins

Chemotaxis

Phagocytosis

Bactericidal Mechanisms

Oxygen-dependent Mechanisms

Oxygen-independent Mechanisms

Neutrophil-Mediated Amplification of Inflammation

Role of Neutrophils in the Adaptive Immune

Response

Neutrophil-Mediated Tissue Injury

Neutrophil-Mediated Injury at Sites of Inflammation

Ischemia/Reperfusion Injury

Neutrophil-Mediated Injury at Distant Sites

Exercise

Inflammatory disease

Equine laminitis

Equine colic

Acute respiratory distress syndrome and multiple organ failure

Role of neutrophils in microvascular thrombosis

Acronyms and Abbreviations

ARDS, acute respiratory distress syndrome; BPI, bacteria permeability-enhancing factor; C, γ subfamily of chemokines; CC, β subfamily of chemokines; CXC, α subfamily of chemokines; CX₃C, δ subfamily of chemokines; CXCL8, interleukin-8; DC, dendritic cell; MODS, multiple organ dysfunction syndrome; IFN- γ , interferon-gamma; ICAM, intercellular adhesion molecule; IL, interleukin; kDa, kilodalton; LBT, leukotriene; lymphocyte function-associated antigen (LFA-1); MOF, multiple organ failure; NETs, neutrophil extracellular traps; PSGL-1, P-selectin glycoprotein ligand-1; TNF- α , tumor necrosis factor-alpha; VCAM-1, vascular cell adhesion molecule-1.

Neutrophils circulate in the blood for only a few hours, existing either in a circulating or marginated pool. In healthy animals, neutrophils randomly leave the circulation, primarily migrating into the gut, lungs, and skin. This process is essential in preventing bacterial infection. When neutrophil numbers in the blood reach a critically low level (usually <500 segmented neutrophils/ μ L) animals are highly susceptible to bacterial infection. Neutrophils also are recruited from the blood to sites of infection. When they encounter a microorganism, they phagocytize and attempt to kill the pathogen. This chapter provides an overview of the mechanisms of neutrophil adherence, chemotaxis, phagocytosis, and organism killing.

ADHERENCE

The recruitment of neutrophils to sites of tissue injury and infection is a hallmark of the acute inflammatory response. Neutrophils leave the flowing bloodstream

by first tethering and then rolling on inflamed endothelium. In the systemic circulation, this occurs primarily in post-capillary venules and collecting venules.²⁴ With the appropriate chemoattractants, rolling neutrophils will become firmly adherent to the endothelial surface and migrate across the endothelium, with transmigration being completed in less than 2 minutes.² Neutrophil transendothelial migration can occur through distinct routes, either paracellular (between endothelial cells) or transcellular (through endothelial cells).

Selectins

Selectins mediate the initial interactions between neutrophils and endothelium, resulting in their attachment and rolling. Rapid on and off bonding events between selectins and their ligands cause leukocytes attached to endothelium to roll under blood flow conditions (Fig. 41.1). There are three known selectins, each with a highly specific pattern of cellular expression. L-Selectin (CD62L) is expressed by leukocytes, E-selectin (CD62E)

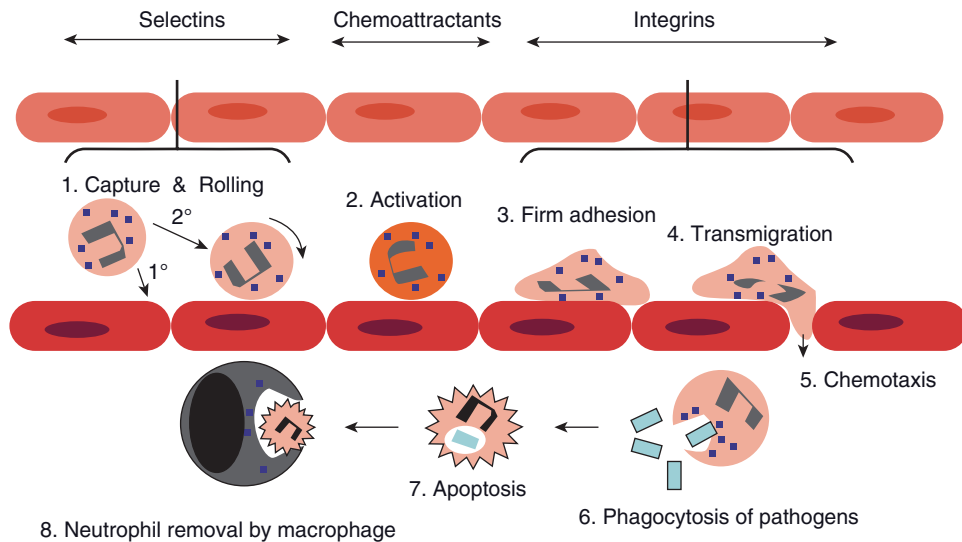


FIGURE 41.1 Antimicrobial activity of neutrophils is a multistep process. Neutrophils in flowing blood accumulate first by direct capture. The tethered neutrophils then roll and become activated by chemoattractants. This leads to firm adhesion and transendothelial migration. Upon entering the tissues, neutrophils migrate toward chemotactic gradients and phagocytose and kill bacterial pathogens. Phagocytosis induces programmed cell death (apoptosis) with resultant phagocytosis by macrophages.

is expressed by endothelial cells, and P-selectin (CD62P) is expressed by platelets and endothelial cells.

The selectins are type I integral membrane proteins composed of distinct tandem protein domains. Neutrophils uniformly and constitutively express L-selectin which plays a broad role in neutrophil recruitment. L-Selectin is tightly regulated. For instance, neutrophils down-regulate L-selectin expression by ectodomain proteolysis (shedding) in a very efficient and rapid manner upon their stimulation by cytokines and chemoattractants.²⁸ Studies performed *in vitro* and *in vivo* indicate that L-selectin shedding affects leukocyte adhesiveness and recruitment.²⁸ L-Selectin shedding maintains high levels of soluble L-selectin in the serum,¹¹ which can diminish leukocyte-endothelium interactions. Soluble L-selectin may serve as an adhesion buffer to suppress neutrophil accumulation below a certain inflammatory threshold. Interestingly, a reduced level of serum L-selectin correlates with susceptibility to inflammatory disease.

The selectin family of adhesion proteins are highly selective lectins that recognize specific glycan structures displayed in most cases on an appropriate protein backbone. P-Selectin glycoprotein ligand-1 (PSGL-1) is the main glycoprotein ligand on neutrophils for P-selectin. PSGL-1 also serves as the primary ligand on endothelium for L-selectin.²⁸

Integrins

Integrins mediate firm adhesion of neutrophils and locomotion by interacting with ligands on cells and in the extracellular matrix (Fig. 41.1). Integrins consist of noncovalent heterodimers (α and β subunits) of type 1 transmembrane glycoproteins, and are made up of subfamilies that are organized around a particular subunit.¹⁰

The β_2 (CD18) subfamily is expressed on all cells of hematopoietic origin and plays a critical role in neutrophil transendothelial cell migration and in phagocytosis and apoptosis. CD18 integrins exist in an inactive state on circulating leukocytes, but undergo rapid affinity and valency changes required for optimal integrin function when stimulated with various chemoattractants. There are four known α (CD11) subunits that can combine with the CD18 subunit to form unique receptors. The α -subunits are defined as CD11a, b, c, and d. The α and β subunits are expressed from different genes and can be independently expressed on different cell types.

CD11b/CD18 ($\alpha_M\beta_2$ or Mac-1) is constitutively expressed by neutrophils, monocytes, and macrophages. Mac-1 is stored in secretory vesicles in neutrophils that are translocated to the surface following certain types of activation, allowing increased Mac-1 cell membrane expression. Mac-1, like LFA-1, is less active on resting cells than activated cells. Mac-1 interacts with intercellular adhesion molecules (ICAMs) and a growing list of other ligands. ICAMs are type I transmembrane glycoproteins and belong to the immunoglobulin superfamily.¹² ICAM-1 is expressed on resting endothelium and is induced on many cell types by inflammatory cytokines. Mac-1 participates in a number of adhesion reactions where the nature of the ligand is not known, such as adhesion of neutrophils to plastic or glass surfaces and binding to a variety of denatured proteins, suggesting a scavenger receptor-like function. Of particular importance is that Mac-1, also referred to as complement receptor 3, binds to C3bi. This complement fragment bonds to surfaces when triggered by immunoglobulin or microbial surfaces (opsonization), and particles coated with C3bi are readily phagocytized by neutrophils.

CD11a/CD18 ($\alpha_1\beta_2$ or lymphocyte function-associated antigen; LFA-1) is the most broadly expressed leukocyte integrin. It is expressed on early hematopoietic progenitor cells and on all mature leukocytes. LFA-1 interacts with ICAM-1, and ICAM-2, which are mainly expressed on endothelium, and ICAM-3 which is mainly expressed on leukocytes.

CD11c/CD18 ($\alpha_x\beta_2$ or P150,95) is highly homologous to Mac-1, but is expressed at lower levels on neutrophils. Like Mac-1, CD11c/CD18 binds to some of the same ligands, including C3bi, fibrinogen, a variety of denatured proteins, as well as binding to many pathogens.¹²

CD11d/CD18 ($\alpha_D\beta_2$) is the most recently defined member of the leukocyte integrin family and is expressed on tissue macrophages, dendritic cells, and eosinophils and binds to ICAM-3 and vascular cell adhesion molecule-1 (VCAM-1).⁵ The latter binding interaction may contribute to recruitment of eosinophils to inflamed airways.

CHEMOTAXIS

Chemotaxis is accomplished by binding of chemokines to neutrophil receptors. This chemokine system is complex involving greater than 50 distinct chemokines and 20 G-protein-coupled chemokine receptors.^{6,9,21} These chemokines are produced either constitutively or in response to inflammatory stimuli and regulate hematopoiesis, leukocyte trafficking, angiopoiesis, tissue architecture, and organogenesis. This diverse response of cells to chemoattractants is largely due to the cells' capacity to express a unique combination of chemokines receptors and the complex network of intracellular signaling that can result from receptor stimulation.

Chemokines are a superfamily of 8- to 15-kDa polypeptide molecules.^{6,9,21} The chemokines are clearly distinct from other chemoattractants, such as complement components C3a and C5a and platelet-activating factor and leukotriene B₄. Based on the presence of a cysteine at the amino terminal, chemokines have been classified into four subfamilies. The α subfamily has two cysteines separated by a single amino acid and so is designated the CXC subfamily. The δ subfamily has two cysteines and is designated the CC subfamily. The γ subfamily has one cysteine and is designated the C subfamily. The δ subfamily has two cysteines separated by three amino acids and is designated the CX₃C superfamily. To unify chemokine nomenclature, chemokines in each subfamily are named with the letter L (to denote ligand) and given a number (e.g. CXCL8 is the new name for IL-8). Although it had been thought that CXC chemokines target neutrophils and CC chemokines target mononuclear cells, more recent data indicate that subfamilies are not target cell-specific. Chemokines are produced by a wide variety of cells in the body. Each family of chemokines interacts with a reciprocal family of heterotrimeric, 7-transmembrane, G protein-coupled receptors that are expressed on leukocytes. In addition

to leukocytes, other cells, including endothelial cells, epithelial cells, and muscle cells express chemokines and their receptors.²⁴ Chemokine receptor expression varies depending on the stage of leukocyte differentiation and exposure to activation stimuli.²⁴ Chemokines bind to receptors on leukocytes causing activation of a variety of cell signaling pathways. This signaling from the chemokine receptor results in polarization, and locomotion, in part through the activation of the integrin adhesion molecules. Leukocyte locomotion towards higher concentrations of a chemoattractant is termed chemotaxis and this can occur in a hierarchical manner. For instance, neutrophils become less sensitive to an initially encountered chemoattractant gradient, allowing them to then respond to a newly encountered chemoattractant.⁸ This process allows neutrophils to find their ultimate target through a complex stimulant environment. Different classes of chemoattractants include chemokines, lipid mediators, complement factors, and other peptides.

PHAGOCYTOSIS

Neutrophils ingest bacteria and other particulate material by receptor mediated phagocytosis.²² This process forms a vesicle around the organism and internalizes it. After internalization, the vesicle containing the organism undergoes a stepwise process of maturation in which the vesicle fuses predominately with primary granules and to a lesser extent with secondary granules. Within the phagolysosome, organisms are digested by proteolytic enzymes and reactive oxygen radicals. The process of phagosome fusion with primary granules and subsequent organism killing is complex and incompletely understood.

BACTERICIDAL MECHANISMS

Antimicrobial mechanisms within the mature phagosome have generally been divided into oxygen-dependent and oxygen-independent mechanisms.¹³ Although these mechanisms interact in complex ways, they will be discussed separately.

Oxygen-Dependent Mechanisms

Oxygen-dependent mechanisms are located within primary granules.¹ These mechanisms are initiated by the process of phagocytosis or by perturbation of the cell membrane and are dependent on a membrane-bound nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase. NADPH oxidase consists of five essential protein components. The membrane-associated component is a flavocytochrome b₅₅₈. Flavocytochrome b₅₅₈ is distributed in the cell membrane and in the membrane of the granules and is incorporated into the wall of phagocytic vacuoles. The cytosolic components consist of a 40-kDa protein (p40-*phox*), a 47-kDa protein (p47-*phox*) and a 67-kDa

component (p67-*phox*). When stimulated, the cytosolic components are translocated to the membrane. Both assembly of the NADPH oxidase complex and electron flow are dependent on the influence of 3 GTP-binding proteins. The NADPH oxidase complex catalyzes the reduction of molecular oxygen to superoxide anion ($\text{NADPH} + \text{O}_2 \rightarrow \text{NADP}^+ + \text{H}^+ + \text{O}_2^-$). The associated rapid consumption of oxygen has been termed the “respiratory burst.” Superoxide anions are rapidly dismutated to hydrogen peroxide (H_2O_2). Superoxide anions also can be converted to hydroxyl radicals (OH^\bullet) in the presence of catalytic metals such as iron according to the Haber-Weiss reaction ($\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^\bullet + \text{O}_2$). Myeloperoxidase catalyzes the conversion of H_2O_2 and halide ions to hypohalous acids with hypochlorous acid being the primary acid produced. Finally, H_2O_2 interacts with hypohalous acids to produce singlet oxygen ($^1\text{O}_2$). Singlet oxygen is a high energy form of oxygen that can attack double bonds. These reactive oxygen intermediates interact with unsaturated lipids, carbon bonds, sulfhydryl and amino groups, nucleic acids, pyrimidine nucleotides, and enzymes within organisms.

Products of the respiratory burst are toxic to cells as well as invading organisms. A variety of antioxidant systems exist to protect cells from the harmful effect of these oxidants.²⁷ These systems include cytosolic superoxide dismutase, glutathione peroxidase, glutathione reductase, and catalase, all of which convert oxygen radicals to water. Other compounds with oxygen radical scavenging potential include vitamin E, vitamin C, selenium, transferrin, and cysteine.²⁷ The glutathione needed for the reaction is maintained in the reduced state by reduced NADPH that is supplied by the pentose phosphate pathway.

Oxygen-Independent Mechanisms

Non-oxidative mechanisms may be more important in killing microorganisms than oxidative mechanisms. Some investigators hypothesize that the major role of the NADPH oxidase system is to adjust phagosomal pH and to pump electrons into the phagocytic vacuole.²⁵ This movement of ions across the phagosome membrane produces conditions that are conducive to oxygen-independent organism killing. For example, gp91^{phox} of NADPH oxidase functions as a proton channel. This proton pump appears to be critical in shuttling hydrogen ion and several other ions across the phagosomal membrane. An influx of potassium appears to be important in liberating proteases such as elastase and cathepsins from their acidic proteoglycan matrix within granules. NADPH oxidase also is thought to induce depolarization that inhibits calcium influx into neutrophils.

Oxygen-independent bacterial killing mechanisms are present within primary, secondary, and tertiary granules. Contents of these granules are discussed in Chapter 40.

Defensins are a family of low-relative-molecular-mass antimicrobial peptides that have been isolated

from neutrophils and heterophils of humans, rats, guinea pigs, and rabbits.⁷ They contain six invariant cysteine residues and are arginine rich. The cysteine residues form disulfide bonds, and pairing of the first and sixth residues creates a cyclic structure. Defensin-like peptides have been isolated from cow and horse neutrophils and chicken heterophils. These peptides have a broad spectrum of antimicrobial activity, being active against both Gram-positive and Gram-negative bacteria, as well as some fungi, enveloped viruses, protozoa, and cells.

Bacteria permeability-enhancing factor (BPE) is a large, lysine-rich protein that is active against *Escherichia coli* and *Salmonella typhimurium* but lacks antimicrobial activity against Gram-positive bacteria and fungi. Bacteria permeability-enhancing factor appears to bind to the outer membrane of susceptible Gram-negative bacteria and increases membrane permeability to hydrophobic molecules.

Granulocytes also contain a variety of proteases and acid hydrolases, the most notable of which are cathepsin G, elastase, collagenase, lysozyme, acid phosphatase, aryl sulfatase, neuramidase, and nuclease. Cathepsin G has microbicidal activity against Gram-positive and Gram-negative bacteria as well as some fungal organisms. Cathepsin G also inhibits bacterial respiration and energy dependent transport systems, as well as protein, DNA, and RNA biosynthesis. Elastase degrades bacterial cell wall proteins and potentiates the lytic activity of lysozyme and the microbicidal activity of cathepsin G. Lysozyme is a cationic protein that hydrolyzes bacterial cell walls by attacking the β 1–4 glycosidic linkage that joins *N*-acetyl muramic acid and *N*-acetyl glucosamine of peptidoglycan. Lactoferrin has bacteriostatic activity that is associated with its capacity to sequester iron. However, lactoferrin also exerts a bactericidal effect independent of its bacteriostatic effects.¹⁶

Neutrophils also appear to trap and kill pathogens in what has been termed neutrophil extracellular traps (NETs).²⁹ Neutrophil extracellular traps form from activated neutrophils and consist of neutrophil-derived DNA embedded with enzymes and antimicrobial peptides. Neutrophil extracellular traps provide a high local concentration of antimicrobial molecules that kill microbes effectively. The process of cell death that results in NET formation is dependent on generation of reactive oxygen species by NADPH oxidase. NETs have been described in cattle.¹⁷ Formation of NETs is not inhibited by milk; therefore NETs may play an important role in defense against mastitis.

NEUTROPHIL-MEDIATED AMPLIFICATION OF INFLAMMATION

Neutrophils secrete a variety of proinflammatory mediators that amplify the inflammatory process. These include leukotrienes, prostaglandins, cytokines, and chemokines. Arachidonic acid and other C20 polyunsaturated fatty acids are the immediate precursors of prostaglandins and leukotrienes (LBTs). They are

released from phospholipids by the action of phospholipase A, phospholipase C, and diacylglycerol lipase. Neutrophils, monocytes, macrophages, eosinophils, and mast cells are major producers of LTB. Separate pathways synthesize LTB₄ and sulfidopeptide leukotrienes (LTC₄, LTD₄, LTE₄). Neutrophils synthesize mostly LTB₄. LTB₄ has both priming and direct activating effects on neutrophils. It is a potent chemotactic and chemokinetic agent for neutrophils, monocytes, and eosinophils. It also stimulates neutrophil aggregation, superoxide production, and integrin expression.

Neutrophils secrete a broad spectrum of cytokines that are mostly involved in the innate immune response.²⁶ Activated neutrophils release tumor necrosis factor- α (TNF- α), IL-1 β , and IL-6. At sites of inflammation, release of these cytokines amplifies the inflammatory process. Neutrophils also produce interferon-gamma (IFN- γ), IL-12, and transforming growth factor- β , known to affect adaptive immunity.

Neutrophils secrete both CC and CXC chemokines. CC chemokines include macrophage inflammatory protein-1 α and -1 β . CXC chemokines include CXCL8 (IL-8) and CXCL10 (IFN- γ -inducible protein-10). Therefore, neutrophils amplify the inflammatory response by attracting more inflammatory cells to the site.

ROLE OF NEUTROPHILS IN THE ADAPTIVE IMMUNE RESPONSE

Although neutrophils are key players of the innate immune response that provide a first line of defense against invading pathogens, there is increasing evidence for their role in modulating the adaptive immune response as well. Neutrophils play an immunoregulatory role and are able to signal other members of the immune system. Through release of chemokines such as MIP-1 α , neutrophils recruit immune cells including T cells, monocytes, macrophages, and dendritic cells (DCs). In addition, antimicrobial peptides produced by neutrophils (e.g. alpha-defensins) can attract T cells and immature DCs. Conversely, regulatory T cells can secrete cytokines (e.g. IL-17) that attract neutrophils. Further, a recent report has demonstrated that regulated cellular interactions between neutrophils and immature DCs contribute to neutrophil-induced adaptive responses. Together, these chemokines, cytokines, and cellular interactions indicate the role of neutrophils in passing information on to other players of the adaptive immune response.

Consistent with these observations are studies that suggest that, in addition to DCs, neutrophils may shuttle particulate antigens and microbes from the periphery to lymphoid tissue. It is unknown how neutrophils that gain access to lymphoid tissue affect the ensuing immune response. It is important to note that most of this information is derived from human and mouse studies and functional differences between neutrophils of human, murine, and veterinary species may limit direct extrapolation of these results.

NEUTROPHIL-MEDIATED TISSUE INJURY

Neutrophils have the capacity to injure host tissues by the same mechanisms as are used to kill invading organisms.^{14,15} This injury can vary from local tissue injury at a site of inflammation to a systemic inflammatory response syndrome. Several experimental models of neutrophil-mediated tissue injury have been developed. These include neutrophil-mediated rat lung injury induced by thermal injury to skin, ischemia-reperfusion-induced injury to several organs, endotoxin-induced lung injury, and injury of isolated perfused organs. Neutrophils have been incriminated in local injury at sites of inflammation and in ischemia-reperfusion injury. Systemic disease states in which neutrophil-mediated tissue injury has been incriminated have variously been termed systemic inflammatory response syndrome, acute respiratory distress syndrome (ARDS), endotoxic shock, septic shock, and multiple organ dysfunction syndrome (MODS).^{14,15} The lungs are particularly sensitive to neutrophil-mediated injury both because of their extensive capillary network and a unique sensitivity of their capillary endothelium to neutrophil-mediated injury. However, other organs, including heart, liver, and kidneys, can be damaged and undergo functional compromise.

Neutrophil-Mediated Injury at Sites of Inflammation

The most studied localized neutrophil-mediated organ injury in veterinary medicine is lung injury associated with shipping fever in cattle. Invasion of the causative organism, *Mannheimia haemolytica*, results in rapid recruitment of neutrophils into the lungs within the first 2 hours. These changes are characterized histopathologically by a fibrinopurulent pneumonia and by endothelial disruption and alveolar epithelial swelling.³⁵ The endothelial and alveolar epithelial damage occurs immediately beneath sites of neutrophil attachment. Activation of neutrophils while they are attached to endothelium may cause vascular injury and resultant increased permeability, resulting in flooding of alveoli with plasma.¹⁸ Neutrophil depletion before experimental inoculation of *Mannheimia haemolytica* organisms into the lungs of calves markedly attenuates alveolar capillary endothelial injury in the first 6–8 hours after inoculation of the organism.

Ischemia/Reperfusion Injury

Prolonged ischemia results in metabolic and ultrastructural changes within cells. Ischemia decreases cellular oxidative phosphorylation resulting in failure to resynthesize high energy phosphate compounds such as adenosine 5'-triphosphate. Adenosine 5'-triphosphate catabolism during ischemia results in accumulation of hypoxanthine. Upon reperfusion of hypoxic tissue, hypoxanthine is converted to oxygen radicals. Ischemia also promotes expression of leukocyte and platelet adhesion receptors on the cell surface, promotes expres-

sion of proinflammatory cytokines, and bioactive agents, and suppresses anti-inflammatory and anti-thrombotic gene products, including nitric oxide, nitric oxide synthase, thrombomodulin, and prostacyclin. Reperfusion injury is primarily directed at the endothelial cells lining the microvasculature and is manifest as accelerated production of a variety of bioactive agents and expression of adhesion molecules for neutrophils.⁴ Neutrophils become activated while attached to endothelium, releasing oxygen radicals and granule contents directly onto the endothelial surface. Superoxide is also generated from hypoxanthine within endothelial cells and by mast cells and macrophages.

Neutrophils may also contribute to tissue hypoperfusion after blood flow is reestablished. Activated neutrophils are rigid and tend to lodge in capillaries. Neutrophil-neutrophil aggregates and platelet-neutrophil aggregates may plug capillaries, resulting in prolonged maldistribution of blood flow. Platelet-neutrophil aggregates may also contribute to microthrombus formation.

Neutrophil-Mediated Injury at Distant Sites

Neutrophils can become activated within the circulation during a variety of physiologic and pathologic conditions in domestic animals. Circulating activated neutrophils have been documented during strenuous exercise, inflammatory disease, equine colic, and equine laminitis. The extent to which these activated neutrophils induce organ injury/failure is uncertain but there is evidence to suggest that these activated neutrophils may contribute to the pathogenesis of organ injury in some disease processes.

Exercise

Strenuous exercise has been associated with up-regulation of neutrophil surface-associated integrin receptors in dogs undergoing short duration sled-pulling activity.²⁰ Additionally, decreased neutrophil granularity was observed post-exercise. Because CD11b/CD18 is stored in neutrophil granules and is transported to the cell surface when neutrophils degranulate, the combination of increased cell surface integrin concentration and decreased granularity post-race is consistent with exercise-associated neutrophil degranulation.

Inflammatory Disease

Neutrophils circulate in an activated state in a variety of inflammatory conditions. Dogs with naturally occurring septic and non-septic inflammatory diseases had increased cell surface expression of CD11b.³⁴ Neutrophils from dogs with septic inflammatory diseases and those with evidence of multiple organ dysfunction also had decreased neutrophil granularity and increased neutrophil size. However, leukocytes may have decreased bactericidal activity. Monocytes and lymphocytes from dogs with septic and non-septic inflammation and multiple organ dysfunction had decreased expression of

major histocompatibility factor class-II, suggesting impaired immune responsiveness.³⁴

Leukocyte deformability, neutrophil CD11b expression, and neutrophil size were evaluated in calves experimentally inoculated intrabronchially with *Mannheimia haemolytica* organisms.¹⁹ Infected calves had decreased leukocyte deformability and increased neutrophil size by 1 hour and increased CD11b expression by 6 hours after organism inoculation. Decreased neutrophil deformability and increased size may contribute to sequestration of neutrophils in alveolar capillary beds during sepsis.

Equine Laminitis

Neutrophils may become activated in the prodromal stages of equine laminitis. At various times during the first 12 hours after induction of black walnut-induced laminitis, horse neutrophils had increased oxygen radical production and some horses had increased phagocytosis of bacteria.³ These changes were associated with neutropenia and were presumed to be due to a systemic inflammatory response resulting from absorption of inflammatory mediators from the intestine. These activated neutrophils have been incriminated in the pathogenesis of the laminar injury.³

Equine Colic

Activated neutrophils were not detected in healthy horses or horses with impaction or gas colic.³⁰ Conversely, horses with inflammatory bowel disease consistently had increased neutrophil cell membrane CD11/CD18 expression, increased neutrophil size, and decreased neutrophil granularity consistent with circulating activated and degranulated neutrophils. These horses also had decreased leukocyte deformability, indicating that neutrophils were rigid. Horses with strangulating colic had variable results. Among horses with strangulating colic, changes in leukocyte deformability, neutrophil size, and neutrophil granularity correlated directly with adverse outcome.

Acute Respiratory Distress Syndrome and Multiple Organ Failure

Activated neutrophils are central to the pathogenesis of ARDS and MODS associated with sepsis and endotoxemia.¹⁵ In these conditions microvascular injury is the major site of organ injury. The lungs are particularly sensitive to neutrophil-induced vascular injury. The process of neutrophil sequestration in the lungs differs from that of other tissues. In other tissues, neutrophil sequestration occurs predominately in post-capillary venules and is dependent on selectin and integrin adhesion molecules. By contrast, neutrophil sequestration in the lungs occurs primarily in pulmonary capillaries and is largely independent of adhesion molecules. Sequestration is thought to be the result of rigid activated neutrophils lodging in the extensive capillary beds within lung tissue.

Limited neutrophil activation while in contact with the endothelium is part of the normal adhesion and emigration process. However, excessive activation of neutrophils while attached to endothelium can result in direct release of oxygen radicals and granule contents onto the endothelial surface. Mediators implicated in various experimental models of ARDS include oxygen radicals, activated complement factors, nitric oxide, proteolytic enzymes, and metalloproteinases.¹⁴ Consequences of this endothelial injury include: (1) massive leakage of plasma into alveoli, (2) vasodysregulation leading to maldistribution of blood flow, and (3) disturbances of oxygen transport and utilization. All of these lead to dyspnea and hypoxemia.

Neutrophils have been incriminated in the pathogenesis in acute inflammatory liver disease.²³ Neutrophils can be activated in the circulation by a variety of factors including cytokines, chemokines, and complement. After activation they accumulate in the hepatic microvasculature and transmigrate into the hepatic parenchyma. Neutrophils can directly adhere to hepatocytes and kill them through oxidative and non-oxidative mechanisms.²³

Role of Neutrophils in Microvascular Thrombosis

Local and disseminated activation of coagulation frequently accompanies sepsis and endotoxemia.³³ The mechanism responsible for this sepsis-induced procoagulant effect is complex. The role of neutrophils in microvascular thrombosis appears to be in their capacity to induce endothelial injury and to bind and activate platelets. Endothelial injury results in conversion of the endothelial cell surface from an anticoagulant surface to a procoagulant surface. Destruction of endothelial cells activates the intrinsic clotting system through exposure of subendothelial collagen and activates the extrinsic coagulation system (i.e. tissue factor pathway) through contact with tissue factor.

Platelets frequently become activated during sepsis and endotoxemia. Both activated neutrophils and activated endothelial cells express platelet-activating factor that is a potent platelet agonist. When activated platelets degranulate, P-selectin is transported from alpha granules to the cell membrane. P-Selectin interacts with PSGL-1 on the neutrophil surface forming platelet-neutrophil aggregates. Platelet-neutrophil aggregates are rigid and, therefore lodge in capillary beds and initiate microvascular inflammatory and thrombotic events. Platelet-neutrophil aggregates have been detected in horses undergoing near-maximal treadmill exercise and in horses and ponies with carbohydrate overload-induced laminitis.^{31,32}

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Neutrophil Function Disorders

DOUGLAS J. WEISS

Congenital Neutrophil Function Defects

- Leukocyte Adhesion Deficiency
 - Bovine leukocyte adhesion deficiency
 - Canine leukocyte adhesion deficiency
- Granule Alterations
 - Chédiak-Higashi syndrome
 - Birman cat neutrophil granulation anomaly
 - Mucopolysaccharidosis
- Defects in Oxidative Metabolism
 - Chronic granulomatous disease in Doberman pinschers
 - Myeloperoxidase deficiency in cyclic hematopoiesis of gray collies
 - Altered chemiluminescence in Weimaraners

Other Defects

- Cyclic hematopoiesis in gray collies
- Pelger-Huët anomaly

Acquired Neutrophil Function Defects

- Neutrophil Dysfunction in Periparturient Dairy Cattle
- Neutrophil Dysfunction in Neonatal Animals
- Neutrophil Dysfunction Associated with Viral Infection
- Neutrophil Dysfunction Associated with Parasitic Diseases
- Effects of Stress on Neutrophil Function
- Effects of Nutrition on Neutrophil Function

Acronyms and Abbreviations

BLAD, bovine leukocyte adhesion deficiency; BVDV, bovine viral diarrhea virus; CD, cluster of differentiation; CLAD, canine leukocyte adhesion deficiency; IBRV, infectious bovine rhinotracheitis virus; Ig, immunoglobulin; LAD, leukocyte adhesion deficiency; MPS, mucopolysaccharide; MSP, mucopolysaccharidosis.

CONGENITAL NEUTROPHIL FUNCTION DEFECTS

Leukocyte Adhesion Deficiency

Leukocyte adhesion deficiency (LAD) syndromes (LAD-1, LAD-1 variants, and LAD-2) result in impaired extravasation of neutrophils into sites of inflammation due to dysfunctional selectin- or integrin-mediated adhesion events.² The LAD syndromes are relatively uncommon; however, the clinical consequences are often severe and lethal, and include recurrent or unresolved localized infections, systemic sepsis, and impaired wound surveillance and repair.

Classical leukocyte adhesion deficiency type 1 (LAD-1) is an autosomal recessive disorder arising from germ line mutations in the gene encoding CD18 and thus affects all CD18 subfamilies.² LAD-1 has been described in humans, Holstein cattle, dogs, and mice.^{2,19} In humans, the CD18-integrin subunit is either not synthesized or unable to associate with the respective CD11

subunits, and the resulting LAD can be characterized as severe to moderate.

Bovine Leukocyte Adhesion Deficiency

Bovine leukocyte adhesion deficiency (BLAD) has been extensively investigated.¹ The molecular basis of BLAD is a single point mutation that results in an aspartic acid to glycine substitution at amino acid 128 of the CD18 polypeptide. Interestingly, expression of L-selectin on neutrophils from BLAD calves appears to be reduced as well. Neutrophil adhesion is markedly impaired and chemotactic responses are diminished. The hallmark clinical findings in BLAD include recurrent or chronic diarrhea, chronic pneumonia, ulcerative stomatitis, gingivitis, chronic dermatitis, stunted growth, and impaired wound healing. Persistent and severe neutrophilia is the most prominent hematologic finding; however, neutrophil morphology is normal. Pathologic lesions are primarily located in lungs and intestinal tract. Neutrophils are present in lung lesions but not in

intestinal ulcers or lesions in other tissues. Therefore, neutrophils can enter the lungs by integrin-independent processes.¹

Canine Leukocyte Adhesion Deficiency

Canine leukocyte adhesion deficiency (CLAD) occurs in Irish Setter and Irish Setter-cross-bred dogs.^{17,19,39} As with BLAD, the molecular basis of CLAD is due to a single point mutation; however, in the dog, a cysteine at amino acid 36 is substituted for a serine. This cysteine is a highly conserved residue in the extracellular domain of CD18, and the structural defect results in a failure to express the CD11/CD18 complex on the leukocyte surface. Affected dogs develop severe bacterial infections shortly after birth and usually die or are euthanized.

Granule Alterations

Chédiak-Higashi Syndrome

Defective phagosome maturation occurs in Chédiak-Higashi syndrome which is inherited in an autosomal recessive manner. Chédiak-Higashi syndrome has been described in human beings, Hereford, Brangus, and Japanese black cattle, Persian cats, beige mice, Arctic foxes, mink, and a killer whale.^{3,11} These species have abnormal lysosomal granule formation and altered degranulation. This appears to result from a defect in lysosomal docking proteins. Granule membrane fusion is defective and discharge of granule contents into the phagosome is delayed.³¹ Additionally, in some species altered microtubule formation results in decreased chemotaxis. A hallmark of the condition is fusion of granules in neutrophils, eosinophils, epithelial cells, renal tubular cells, and Kupffer cells. Resultant pink- to magenta-staining granules are seen in all species (Fig. 42.1). Neutrophil function abnormalities vary somewhat between species. Cattle and mice appear to have increased susceptibility to bacterial infection as a result

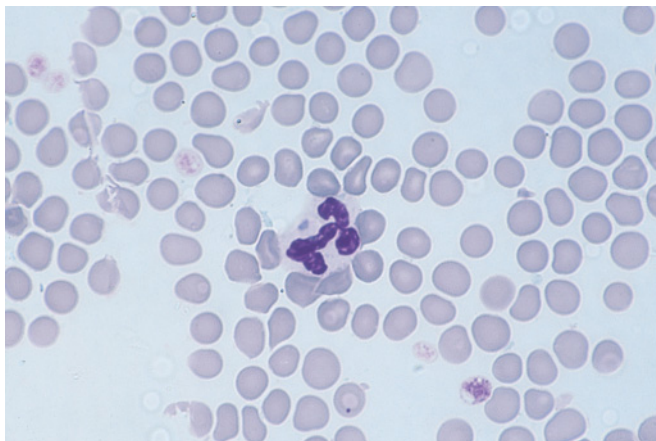


FIGURE 42.1 Giant lysosomes in the neutrophil cytoplasm of a cat that has Chédiak-Higashi.

of impaired neutrophil function.^{33,44} Neutrophil function appears to be less severely affected in cats.¹¹ Most species have platelet-related bleeding due to platelet granule defects.³³ Humans and animals have partial oculocutaneous albinism due to altered distribution of melanin granules. Typical of the pigmentation alterations are the beige mouse, the Aleutian mink, and the smoke blue Persian cat with yellow-green irises. Diagnosis of Chédiak-Higashi syndrome is based on the presence of altered pigmentation, presence of giant granules, hemorrhagic tendencies, and possible susceptibility to infection.

Birman Cat Neutrophil Granulation Anomaly

A granulation alteration in neutrophils is inherited as an autosomal recessive trait in Birman cats.²³ The anomaly is characterized by fine eosinophilic-stained granules in Romanowsky-stained blood smears (Fig. 42.2). In bone marrow, affected granules stain similar to granules present in progranulocytes. These granules do not stain metachromatically with toluidine blue dye as do granules in animals affected with mucopolysaccharidosis. No alterations in neutrophil ultrastructure have been reported. Additionally, no defects in neutrophil cytochemistry, phagocytosis, oxidative metabolism, or bactericidal activity have been observed. Affected cats do not appear to be predisposed to infections; however, the granules need to be differentiated from toxic granulation and mucopolysaccharidosis types VI and VII.

Mucopolysaccharidosis

Mucopolysaccharidosis (MSP) is characterized by large intensely stained azurophilic granules in neutrophils and bone and cartilage defects.²¹ These granules stain metachromatically when stained with toluidine blue dye. The defect involves enzyme deficiencies in mucopolysaccharide (glycosaminoglycan) catabolism. This results in accumulation of metabolic byproducts within

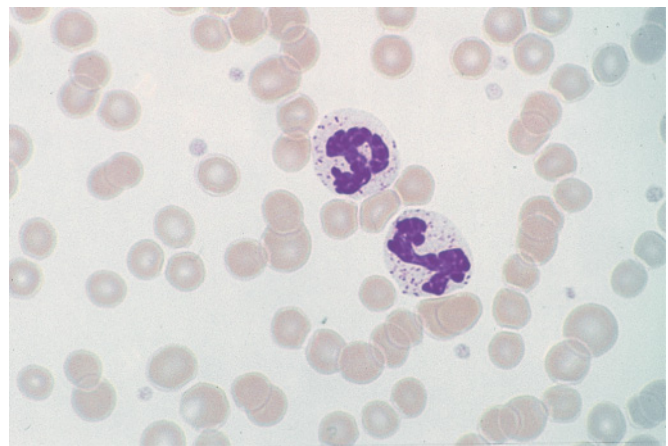


FIGURE 42.2 Birman cat neutrophil granulation anomaly with azurophilic cytoplasmic granules. (Courtesy of Dr. K.S. Latimer, University of Georgia, Athens, Georgia.)

leukocyte granules. Several genetic forms of MSP have been described in cats and dogs including MSP I (Hurler's syndrome), MSP II (Hunter syndrome), MSP III (Sanfilippo syndrome), MSP IV (Morquio syndrome), and MSP VI (Maroteaux-Lamy syndrome), and MSP VII (beta-glucuronidase deficiency). Although neutrophils from affected animals do not appear to have functional defects, the presence of prominent granulation in some cells must be differentiated from other causes of prominent granulation. Differential diagnoses include, Chédiak-Higashi syndrome, Birman cat neutrophil granulation anomaly, and toxic granulation.

Defects in Oxidative Metabolism

Chronic Granulomatous Disease in Doberman Pinschers

Several defects in the capacity of neutrophils to generate oxygen radicals have been described in humans. These include chronic granulomatous disease, glutathione peroxidase deficiency, glucose-6-phosphatase deficiency, and myeloperoxidase deficiency.²⁸ Chronic granulomatous disease is caused by mutations in genes encoding components of NADPH oxidase. In excess of 40 specific mutations have been reported. Chronic granulomatous disease is characterized by recurrent severe, but usually not fatal, bacterial and fungal infections beginning in early childhood.²⁸ Neutrophils have an inability to mount a respiratory burst due to inability to activate the NADPH oxidase system. A neutrophil bactericidal dysfunction with decreased capacity to mount a respiratory burst has been described in eight closely related Doberman pinschers.⁵ These dogs all had a history of chronic bacterial pneumonia that improved with antibiotic treatment but relapsed when antibiotics were discontinued. Both Gram-positive and Gram-negative bacteria were cultured from a transtracheal aspirate of one dog. The neutrophils phagocytized bacteria normally but had a defect in oxygen radical generation. This defect was not as severe as seen in humans with chronic granulomatous disease and it is not clear if the defect was in NADPH oxidase or in another pathway such as myeloperoxidase.

Myeloperoxidase Deficiency in Cyclic Hematopoiesis of Gray Collies

Myeloperoxidase deficiency is the most common of the inherited neutrophil defects in humans.²⁸ However, the condition produces few clinical signs. Although killing of bacterial organisms is delayed, killing is eventually complete. Myeloperoxidase deficiency has been described in cyclic hematopoiesis of gray collie dogs.²⁴ Affected dogs have defective bactericidal activity.

Altered Chemiluminescence in Weimaraners

Abnormal neutrophil chemiluminescence responses have been described in a group of Weimaraner puppies.

These dogs had a history that included recurrent fevers, diarrhea, pneumonia, pyoderma, lymphadenopathy, stomatitis, and osteomyelitis.¹² Decreased chemiluminescence in response to phorbol esters was the major neutrophil function alteration noted in these dogs. Although these dogs had lower serum IgG and IgM concentrations, no defects in humoral or cellular immune responses were detected.

Other Defects

Cyclic Hematopoiesis in Gray Collies

Cyclic hematopoiesis is an autosomal recessive disorder characterized by severe cyclic neutropenia, platelet-related bleeding, recurrent bacterial infections, and coat color dilution.¹⁰ Although the human condition results from a mutation in neutrophil elastase, dogs have a mutation of the β subunit of adaptor protein complex 3 (AP3).²⁴ The specific defect is an insertion of an adenine nucleotide residue in exon 20 leading to a frameshift with premature termination and absence of mRNA. An equivalent mutation causes Hermansky-Pudlak syndrome type 2 in humans, pearl mice, and ruby *Drosophila*.²⁴ This rare syndrome is characterized by cutaneous and retinal hypopigmentation and platelet-related bleeding that is associated with abnormal platelet dense granules.

AP3 is a membrane cargo transport protein that is specifically responsible for routing proteins from the Golgi network to lysosomes. AP3 may be a carrier protein for neutrophil elastase. If true, the canine defect would result in mistrafficking of neutrophil elastase.²⁴ If all types of cyclic neutropenia are associated with increased elastase on neutrophil membranes, signaling through elastase may explain the periodicity observed in cyclic neutropenia.¹⁵ Arrest of hematopoiesis occurs at regular 11–14 day intervals. Because neutrophils have the shortest half-life, neutropenia is the most prominent feature in the blood. Neutropenia lasts 3–4 days and is followed by a neutrophilia. Clinically, affected puppies typically die at birth or during the first few weeks of life and rarely live longer than one year. Dogs that survive the neonatal period may be stunted and typically develop recurrent bacterial infections during periods of neutropenia.²⁷ They also develop amyloidosis in multiple tissues with resultant renal disease and coagulopathies. Diagnosis is based on coat color, cyclic variation in neutrophil count, increased susceptibility to bacterial infections, and decreased myeloperoxidase activity in neutrophils.²⁴ Canine G-CSF can be administered to eliminate the cyclic neutropenia.²⁷ Bone marrow transplantation eliminates the cyclic hematopoiesis and is curative.

Pelger-Huët Anomaly

Pelger-Huët anomaly is an inherited condition identified in dogs, cats, horses, rabbits, and humans and is characterized by failure of mature granulocyte nuclei

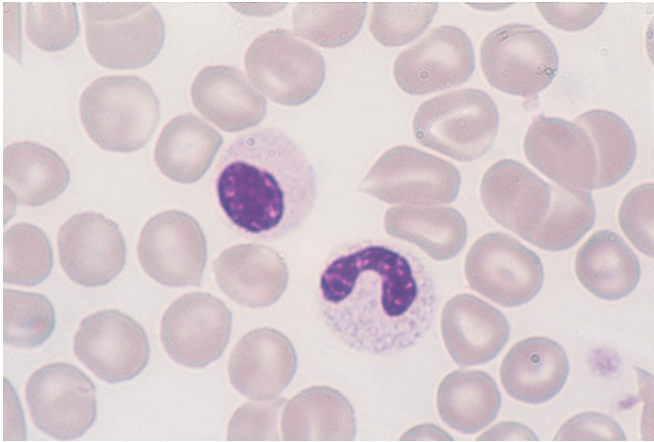


FIGURE 42.3 Band and myelocyte neutrophils from a border collie that has Pelger-Huët anomaly. (Courtesy of Dr. K.S. Latimer, University of Georgia, Athens, Georgia.)

to lobulate (Fig. 42.3).¹⁸ Megakaryocytes are also hyposegmented suggesting that this is a stem cell defect. The condition appears to be inherited in most species as an autosomal dominant trait. The human and mouse mutation is caused by a mutation in the gene encoding for lamin B receptor.⁴² Apparently nuclear lamins are involved in segmentation of granulocyte nuclei.

Heterozygote dogs do not have signs of disease and the condition is usually an incidental laboratory finding. Granulocytes are hyposegmented but the chromatin is condensed. It is important to differentiate this condition from a left shift associated with inflammatory disease. Pelger-Huët anomaly must also be differentiated from pseudo-Pelger-Huët anomaly which refers to an acquired hyposegmentation of granulocytes secondary to infections, myelodysplastic syndromes, or drug treatments. The main difference is that the chromatin is more clumped in Pelger-Huët anomaly. Although some initial reports indicated decreased neutrophil chemotaxis, more recent reports indicate no alteration in neutrophil function in Pelger-Huët anomaly. Many breeds are affected. The homozygous condition, described in rabbits and a kitten, is associated with skeletal deformities and increased susceptibility to infection and is usually lethal.

ACQUIRED NEUTROPHIL FUNCTION DEFECTS

Neutrophil Dysfunction in Periparturient Dairy Cattle

A variety of studies have documented neutrophil dysfunction in periparturient dairy cattle. In one study, significantly decreased random migration, iodination, and chemiluminescence of neutrophils was observed 1 week after parturition.²⁵ In another study, decreased neutrophil oxidative burst activity was observed between 1 and 3 weeks after calving.¹⁴

The cause of periparturient neutrophil dysfunction is incompletely understood. Selenium and glucocorticoid concentrations in periparturient cattle may be a significant factor in predicting neutrophil function. Neutrophils from post-parturient dairy cows with relatively high blood selenium concentrations had greater superoxide production and greater potential to kill bacteria when compared to cows with relatively low selenium concentrations.⁹ Similar neutrophil function alterations have been reported in periparturient cattle and in glucocorticoid-treated cattle.⁸ This suggests that high glucocorticoid concentration in periparturient cattle may be responsible for the neutrophil function defects.

Altered recruitment of neutrophil to the mammary gland and altered neutrophil function in the mammary gland have been incriminated as factors in increased incidence of mastitis during the periparturient period.^{25,34} In general, neutrophils in milk are less effective than blood neutrophils in phagocytizing and killing bacteria.³⁴ This has been attributed to the lower energy reserve of milk neutrophils and phagocytosis of milk fat globules and casein. Milk has been reported to reduce the capacity of neutrophils to phagocytize bacteria 3-fold.³⁵ Neutrophil recruitment appears to be a critical determinant of resistance to mastitis. Post-parturient cows have a reduced capacity to recruit neutrophils into the mammary gland during coliform mastitis.⁴³

Genetic markers have also been associated with susceptibility to bovine mastitis. Cows with allelic variation in CXCR2 (i.e. the IL-8 receptor) had increased susceptibility to mastitis.³⁸ Cows with a CC or GC at CXCR2 + 777 had decreased neutrophil migration in response to IL-8 when compared to cows expressing the GG genotype. Additionally, decreased upregulation of cell surface CD18 was observed on neutrophils after stimulation with IL-8. The results of these studies support a genetic predisposition to mastitis and further emphasize the importance of rapid mobilization of neutrophils into the mammary gland in prevention of infection.

Neutrophil Dysfunction in Neonatal Animals

Neutrophil dysfunction appears to contribute to the susceptibility of newborn calves and foals to septicemia, endotoxemia, pneumonia, and enteritis. Reports of neutrophil dysfunction in foals are inconsistent. When compared to horses, some reports indicate that foals have no difference in phagocytic activity; others report decreased phagocytosis when organisms are opsonized with autologous serum but not when opsonized with adult serum, while yet others report that phagocytosis of *Staphylococcus aureus* is decreased when opsonized with either autologous or adult serum.³⁰ Bacterial killing has been reported to be reduced in some studies and similar to that of adult horses in other studies.⁴⁵ Other neutrophil function alterations reported in foals include increased random migration, increased CD18 expression, decreased chemotaxis, decreased iodination, and the presence of an unidentified serum factor that suppresses oxidative burst activity.

Reports concerning neutrophil dysfunction in calves also tend to be inconsistent. This is in part due to variation in the age of the calves, the colostrum status, and the techniques used to evaluate neutrophil function. Both increased and decreased respiratory burst activity have been reported in neonatal calves when compared to cows.^{13,22} However, this appears to be agonist-dependent. This suggests that age-related alterations in cell membrane receptors or intracellular signal transduction may be involved in neonatal respiratory burst activity.²² Other functional alterations in neonatal neutrophils include decreased myeloperoxidase and increased alkaline phosphatase activities, decreased concanavalin A capping, increased chemotaxis, decreased Fc receptor number, increased aggregation, and increased shape change.⁴⁷

Neutrophil Dysfunction Associated with Viral Infection

Viral infections have been incriminated as predisposing food animals to bacterial infections. This effect may, in part, be mediated by virus-induced neutrophil dysfunction. The bovine viral diarrhea virus (BVDV) is a classic example of a virus that induces neutrophil dysfunction.⁷ Neutrophils from cows experimentally infected with either noncytopathogenic or cytopathogenic strains of BVDV develop marked impairment of iodination capacity. However, other measures of oxygen radical generation are not altered. Even cattle given modified-live BVDV vaccines have altered neutrophil function.⁴⁰ Cattle that are persistently infected with BVDV have multiple alterations in neutrophil function, including decreased random migration, decreased phagocytosis of *Staphylococcus aureus*, decreased iodination, decreased reduction of cytochrome c, decreased antibody-independent cell-mediated cytotoxicity, decreased cytoplasmic calcium flux, and decreased oxygen radical production.

Another virus incriminated as predisposing cattle to bacterial pneumonia is infectious bovine rhinotracheitis virus (IBRV).^{6,29} In one study, calves were exposed to IBRV 5 days before exposure to *Mannheimia haemolytica*.^{6,29} IBRV-exposed calves had decreased neutrophil recruitment to the lungs. Neutrophils from IBRV-exposed calves had decreased random migration and chemotactic responses. Further, alveolar macrophages had decreased capacity to produce chemotactic factors. The results of this study indicate that IBRV infection may predispose cattle to secondary bacterial infections.

Other bovine virus infections associated with altered neutrophil function include parainfluenza 3 virus, bovine herpes virus 1, and bovine immunodeficiency-like virus.^{6,16} Infection of cattle with parainfluenza 3 virus results in decreased iodination and oxidative metabolism in neutrophils. Infection with bovine herpes virus 1 results in reduced neutrophil random migration but enhanced bacterial ingestion. Infection with bovine immunodeficiency-like virus is associated with decreased neutrophil antibody-independent cell-mediated cytotoxicity and iodination activities.

Neutrophil function has also been investigated in cats infected with feline leukemia virus.²⁶ Neutrophil chemotaxis was evaluated in vitro by use of a modified Boyden chamber apparatus. Cats that were viremic and clinically ill had decreased chemotactic and chemiluminescence responses.

Neutrophil Dysfunction Associated with Parasitic Diseases

Infection of cattle with coccidia and infection of a dog with *Prototheca zopfii* resulted in suppressed neutrophil function.^{37,41} Cattle infected with *Eimeria* sp. had decreased neutrophil oxidative metabolism and iodination activity, and had increased susceptibility to bacterial pneumonia.⁴¹ Serum from a dog infected with *Prototheca zopfii* suppressed neutrophil chemotactic activity.³⁷

Effects of Stress on Neutrophil Function

The effects of environmental, transportation, and exercise stress on neutrophil function have been evaluated. The effect of 4 hours of transportation was evaluated on four 4–6 month old Holstein calves.³² After transportation, calves had a neutrophilia, enhanced neutrophil superoxide production, suppressed lymphocyte blastogenesis and increased plasma cortisol concentrations. These data suggest that neutrophils became activated during transportation despite the potentially suppressive effects of cortisol on neutrophil function.

The effects of cold stress on neutrophil function have been evaluated in bovine neonates.⁴⁶ Exposure of neonatal calves to temperatures of 1°C for 3 days had no effect on bactericidal activity of neutrophils.

The effects of exercise on equine neutrophil function have been evaluated.³⁶ When untrained horses were subjected to moderately intense exercise, an increase in neutrophil oxidative burst activity and neutrophil phagocytosis was observed. However, high intensity exercise was associated with transient impairment of oxidative burst activity and phagocytosis. After 17 weeks of exercise training, neutrophil oxidative burst activity and neutrophil phagocytosis were decreased when compared to neutrophil function test results at 0 and 10 weeks. These data suggest that high intensity exercise training and long duration training have a general suppressive effect on equine neutrophil function.

Effects of Nutrition on Neutrophil Function

Selenium and vitamin E appear to be important nutrients in maintaining neutrophil function. Neutrophils from post-parturient dairy cows with relatively high blood selenium concentrations have greater superoxide production and greater capacity to kill bacteria than those from cows with relatively low blood selenium concentrations.⁹ Injection of selenium-vitamin E into cows has been reported to increase the capacity of

neutrophils to kill bacteria.²⁰ Because selenium is required for glutathione metabolism, selenium deficiency probably causes a defect in the oxygen-dependent pathway of bacterial killing.

Copper deficiency may also adversely affect neutrophil function. Neutrophils from cattle made copper deficient by feeding diets low in copper and high in molybdenum or iron were reported to have decreased capacity to kill *Candida albicans* organisms.⁴

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Eosinophils and Their Disorders

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Regulation, Production, and Release	Phagocytosis
Tissue Distribution and Metabolism	Antitumor Effect
Structure and Morphology	Mechanisms of Eosinophil Effector Function
Surface Molecules	Release of Granule Proteins
Signs of Activation	Release of Lipid Mediators
Involvement in Homeostasis and the Immune System	Cytokine Release
Trafficking in Eosinophilic Inflammation	Mechanisms of Eosinophilia and Eosinopenia
Effector Functions	Eosinophilia
Defense Against Helminthic Parasites	Eosinopenia
Effector Cells in Asthma and Allergic Disease	Therapeutic Approaches
Modulation of Inflammation	

Acronyms and Abbreviations

CLCP, Charcot-Leyden crystal protein; EBP, eosinophilic bronchopneumopathy; ECF-A, eosinophil chemotactic factor of anaphylaxis; ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; EoSV, eosinophil sombrero vesicle; EPO, eosinophil peroxidase; HES, hypereosinophilic syndrome; HETE, hydroxyecosatetraenoic acid; HHT, hydroxyheptadecatrienoic acid; GI, gastrointestinal; IFN, interferon; IL, interleukin; MBP, major basic protein; MCP-3, monocyte chemotactic peptide-3; MIP-1a, macrophage inflammatory protein-1a; PAF, platelet-activating factor; PGE, prostaglandin E; RER, rough endoplasmic reticulum; Siglec, sialic acid-binding Ig-like lectin; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment receptor; TGF, transforming growth factor; WBC, white blood cell.

The eosinophil was named by Ehrlich based on its affinity for anionic dyes, such as eosin.²⁷ This cell defends against helminthic parasites, is a bidirectional partner in basophil- or mast cell-mediated inflammation, and has the potential to damage host tissues. Eosinophils also are important in innate, acquired, and adaptive immunity, tissue remodeling, and developmental biology. No longer regarded simply as an end-stage effector cell, the eosinophil is a pleotrophic multifunctional cell that serves complex physiologic roles. This chapter emphasizes structure–function relationships to explicate the eosinophil’s varying roles in many complex processes. The reader is referred to several recent comprehensive reviews.^{22,28,31,37,38,56}

REGULATION, PRODUCTION, AND RELEASE

Eosinophils develop in bone marrow and to a lesser extent in thymus, spleen, lung, lymph nodes in some laboratory species (see Chapter 7).^{17,29,65} Type 2 helper T

(TH₂) cells, which secrete IL-5 and IL-13, are important regulators of all aspects of eosinophil development and function, including increased production by bone marrow, mediated by IL-5 and recruitment to tissues by eotaxins, regulated by IL-13.

Eosinophils differentiate and mature in bone marrow over 2–6 days, depending on the species. They comprise less than 10% of bone marrow nucleated cells, and the marrow-to-blood eosinophil ratio varies between 300:1 in guinea pigs to 3.4:1 in humans. Generation and emergence times are shortened in animals that have *Trichinella* infections. In rats, eosinophils travel to the spleen to complete maturation. In healthy individuals, the half-life of eosinophils in circulation varies from less than 1 hour in the dog to 18–24 hours in people. Eosinophils migrate into tissues, especially the gastrointestinal (GI) tract and lungs, where they live for about 2 days unless anti-apoptotic factors, such as IL-5, prolong their survival for up to 2 weeks. Under pathologic conditions, it is possible for eosinophils to re-enter circulation.¹³

TISSUE DISTRIBUTION AND METABOLISM

Eosinophils are tissue-dwelling cells, residing predominantly in the skin, respiratory tract, and GI tract. The location and number of eosinophils vary with species, stage of estrous cycle, diet, and histamine content of the tissue; however, the major population of eosinophils is found in the GI tract where they are recruited by the chemokine eotaxin-1 (CCL11).^{42,45}

STRUCTURE AND MORPHOLOGY

Eosinophils are polymorphonuclear leukocytes that become recognizable morphologically with the appearance of specific, or secondary, granules at the progranulocyte stage (Fig. 43.1). Myelocytes have numerous immature specific granules, as well as an extensive Golgi complex and abundant rough endoplasmic reticulum (RER). The nucleus undergoes characteristic segmentation, and granules continue to mature in the band and segmented forms. Eosinophils contain three types of granules, specific granules, primary granules, and small dense granules, as well as sombrero vesicles (EoSVs),⁴⁴ lipid bodies, mitochondria, free ribosomes, sparse RER, a small Golgi, and glycogen.^{2,15}

Specific granules contain potent cytotoxic proteins and comprise the majority of the granule population in eosinophils. They have a bicompartamental structure in many species, including humans, rhesus monkeys, cats, goats, rabbits, opossum, guinea pigs, rats, and mice,^{2,32,43} with a distinctive electron-dense core, or crystalloid, that is characterized by both longitudinal and cross-sectional periodicity and is surrounded by a more lucent matrix (Fig. 43.2 A–D). Cores vary in density, size, and shape among species, and there is especially wide variation in cats. Approximately 10% of canine granules have distinct cores.³⁰ Equine eosinophil granules contain dense cores that lack crystalline patterns, are often located eccentrically, and are surrounded by a less dense matrix.⁵⁵ Homogeneous granules are found in eosinophils from cattle, mink, and gorillas.^{32,43} Features of the four major proteins are described in Table 43.1.

Major basic protein (MBP), located in the core, comprises >50% of the granule protein content. Deposits of MBP can be found in tissues at sites of eosinophilic inflammation. The other proteins are found in the matrix. Eosinophil peroxidase (EPO) can be found in progranulocytes and myelocytes in the RER, perinuclear cisterna, Golgi, and specific granules. Thereafter, EPO is located solely in specific granules.^{2,15} Eosinophil peroxidase is distinct from and more potent than myeloperoxidase.² Different sources conclude that EPO is involved in bacterial killing as well as having activities against parasites and viruses.^{50,60} This enzyme has been widely reported to be absent from feline eosinophils; however, protein peaks separated from feline eosinophil granules are most compatible with EPO, eosinophil cationic protein (ECP), and MBP.²⁰ Further characterization is still needed. ECP constitutes ~30% of

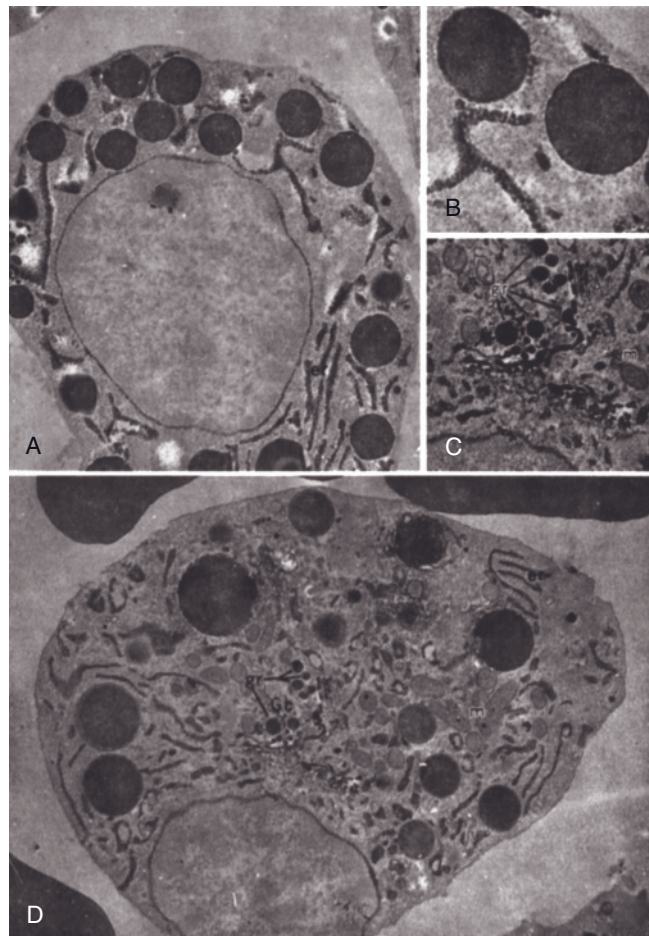


FIGURE 43.1 Eosinophil progranulocyte and myelocyte from canine bone marrow stained for eosinophil peroxidase activity (EPO). (A) A progranulocyte contains dense, homogeneous granules and cisternae of rough endoplasmic reticulum (er), highlighted in (B). The myelocyte shown in (D) contains condensing granules of varying morphology, an active Golgi complex (Gc) producing granules (gr), profiles of rough endoplasmic reticulum, and numerous mitochondria. (C) An enlargement of the Golgi area from the cell in (D). Eosinophil peroxidase activity is characterized by the electron-dense material in the perinuclear cisterna, rough endoplasmic reticulum, Golgi complex, and specific granules. A, 10,080 \times ; B, 18,900 \times ; C, 14,280 \times ; D, 10,080 \times .

the granule protein and damages membranes by a colloid osmotic process, causing formation of transmembrane non-ion-selective pores. Eosinophil cationic protein promotes mast cell degranulation and is bactericidal. Two isoforms have been identified by chromatography.²² Eosinophil-derived neurotoxin (EDN) is also expressed in mononuclear cells and possibly neutrophils. This protein damages myelinated nerve fibers and is implicated in antiviral activity against respiratory viruses.⁵⁰ Other granule constituents include catalase, peroxisomal lipid B oxidation enzymes (enoyl-CoA hydratase, 3-ketoacyl-CoA thiolase), flavoprotein (acyl-CoA oxidase), β -glucuronidase, cathepsin D, serine:pyruvate aminotransferase, and zinc.^{15,32} Acid phosphatase and arylsulfatase activities may be

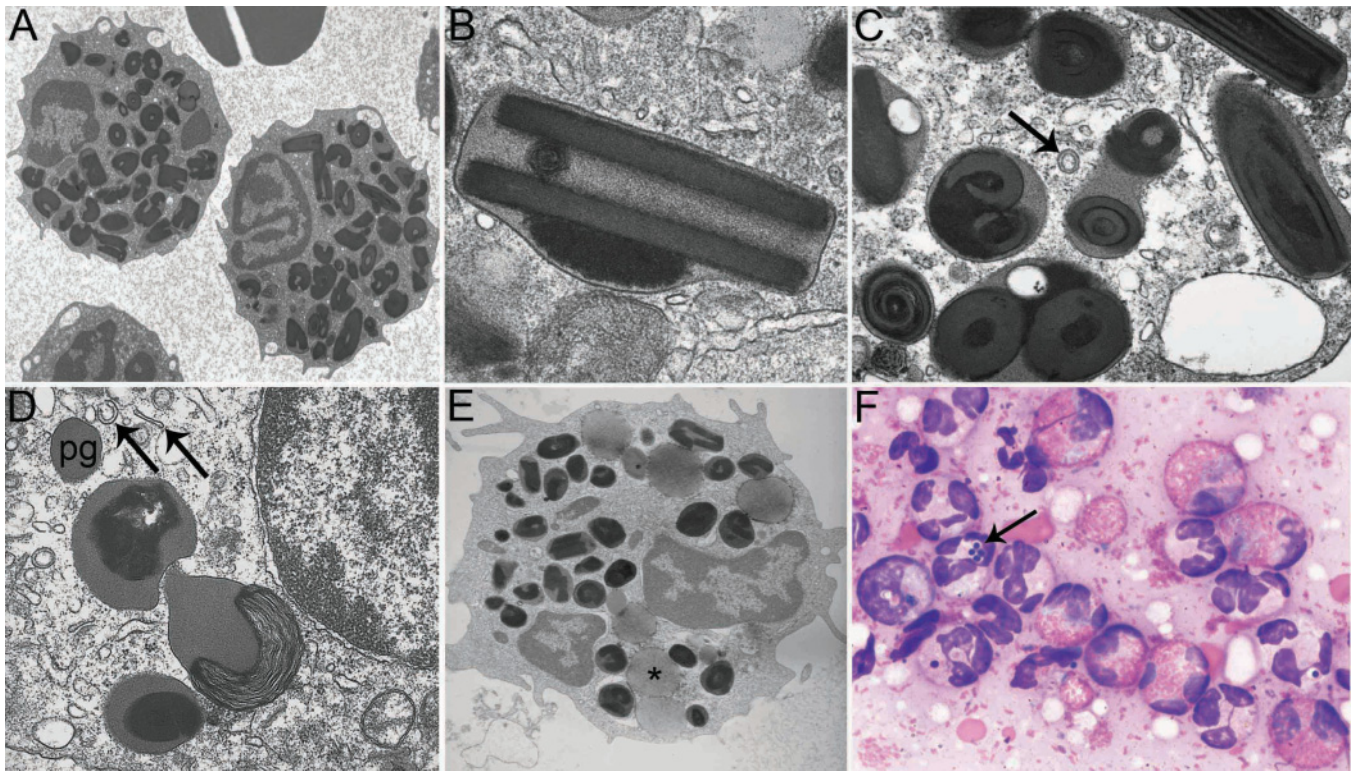


FIGURE 43.2 Transmission electron micrographs (A–E) and cytologic preparation (F) of feline eosinophils in blood and tissue. (A) Two blood eosinophils containing numerous heterogeneous bicompartmental granules. 34,500 \times . (B) A rod-shaped specific granule with electron-dense cores (which may be disassembling) and lucent matrix. 340,000 \times . (C) Multiple specific granules with disassembling electron-dense cores surrounded by more lucent matrix. The arrow indicates an eosinophil sombrero vesicle (EoSV). 340,000 \times . (D) Specific granules, some of which are undergoing piecemeal degranulation. Arrows indicate c-shaped and elongate EoSV that transport and store granule proteins. A primary granule (pg) is present. 340,000 \times . (E) Transmission electron micrograph of an activated eosinophil from a transtracheal wash of a cat with allergic bronchitis. Lipid bodies (asterisk) are increased. 39,100 \times . (F) Eosinophilic inflammation in the skin of a cat with staphylococcal hypersensitivity. A neutrophil contains bacterial organisms (arrow). Wright's stain; 1,000 \times .

unmasked in the matrix when eosinophils are activated.¹⁵ Four basic proteins have been isolated from equine eosinophils but could not be correlated definitively to human proteins, although one is likely the analogue of human MBP.⁴⁸

Membrane-bound primary granules are most numerous in the progranulocyte, but can be found in all stages of eosinophils in some species (Fig. 43.2D). Their lysophospholipase activity has been reported to reside in the Charcot-Leyden crystal protein (CLCP) but may be due to other eosinophil lysophospholipases.²⁸ CLCP comprises 7–10% of total eosinophil protein.^{15,16} This protein polymerizes to form hexagonal crystals deposited in tissues at sites of eosinophilic inflammation even when intact eosinophils are no longer visible. The function of CLCP remains obscure.²⁸ Lysophospholipase dampens inflammation by impeding generation of arachidonic acid metabolites and may inactivate cell-damaging lysophosphatides produced during degranulation.²² A third type of eosinophil granule, the small dense granule, contains acid phosphatase, arylsulfatase, and perhaps ECP, catalase, and peroxidase.

Lipid bodies store arachidonic acid, chiefly in esterified forms, used in the generation of lipid mediators such as leukotrienes and prostaglandins.¹⁵ Eosinophils

also synthesize cytokines, proteoglycans, vitamin B-binding proteins, and numerous enzymes, including collagenase, histaminase, phospholipase D, and non-specific esterases. At least 35 cytokines, chemokines, and growth factors are produced by eosinophils.²⁸ Eosinophils are the chief producers of cytokines such as TGF- β in a variety of eosinophil-associated diseases such as asthma.

The plasma membrane contains nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to generate an oxidative burst and alkaline phosphatase in some species (cattle, horses, dogs, and cats).³² Eosinophils lack the bactericidal agents lysozyme, lactoferrin, and phagocytin.¹⁴

SURFACE MOLECULES

The surface of eosinophils, as visualized by scanning electron microscopy, has short blunt processes and sparse microvilli. Eosinophils have many cell surface receptors including receptors for cytokines, chemokines, complement proteins, immunoglobulins (Ig), adhesion molecules, lipid mediators, enzymes, and signaling and apoptotic factors.⁶⁸ Adhesion receptors link

TABLE 43.1 Summary of Major Preformed Proteins Released from Eosinophil Specific Granules

	Location	Characteristics	Actions
Major basic protein (MBP)	Specific granule core (also found in basophils)	Cationic protein, 14 kDa, pI 10.9, 117 amino acid (aa) single polypeptide rich in arginine; pre-molecule (207 aa) has a pI of 6.2 owing to a 90 aa acidic portion thought to protect cell from toxic effects of MBP during transport	Cytotoxic to parasites, protozoa, bacteria, normal mammalian epithelial cells, murine tumor cells; stimulates signaling pathways and mediator release from mast cells, neutrophils, and basophils; neutralizes heparin; activates platelets, basophils, mast cells, neutrophils; induces bronchospasm; activates complement via classical and alternative pathways; activates remodeling factors from epithelial cells; increases cutaneous vasopermeability; adversely affects smooth muscle contraction responses; implicated in regulating peripheral nerve plasticity by inhibiting apoptosis; no enzymatic activity
Eosinophil peroxidase (EPO)	Specific granule matrix	Heavy (58 kDa) and light (14 kDa) subunits; pI 10.8; genetically and biochemically distinct from myeloperoxidase	In presence of H ₂ O ₂ and halide, generates oxygen radicals toxic to helminths, bacteria, mycoplasmas, fungi, protozoa, viruses, tumor cells; in absence of H ₂ O ₂ acts as cationic toxin; toxic to host respiratory epithelium; induces release of granules, histamine from mast cells; inactivates leukotrienes
Eosinophil cationic protein (ECP)	Specific granule matrix	Cationic protein, 18–21 kDa, pI 10.8, 133 aa; secreted and stored forms differ; ribonuclease gene superfamily; 66% sequence homology with EDN	Toxic to helminths, protozoa, bacteria, tracheal epithelium; neurotoxic; neutralizes heparin; promotes mast cell degranulation
Eosinophil-derived neurotoxin (EDN, protein X, EPX)	Specific granule matrix	Cationic protein, 18–19 kDa, pI 8.9, 134 aa; ribonuclease gene superfamily; 66% sequence homology with ECP	Toxic to myelinated nerve fibers; significant ribonuclease activity; antiviral activity in respiratory infection

eosinophils with endothelial cells or extracellular matrix components, allowing them to leave the circulation and directing their movement in tissues and extracellular spaces. Chemotactic factors and chemokines recruit eosinophils to sites of inflammation. Many factors, including lipid mediators, immunoglobulins, and complement components, act through receptors to activate eosinophils to perform effector functions and to connect them with their targets.^{32,37,38}

More recently, surface molecules previously associated with other cell types have been identified on eosinophils and include Toll-like receptors and inhibitory receptors, such as sialic acid-binding Ig-like lectin (Siglec-8).^{38,51} Activation of inhibitory receptors suppresses eosinophil activation and induces negative signaling in murine eosinophils; cross-binding Siglec-8 induces apoptosis of eosinophils.²⁸

SIGNS OF ACTIVATION

Activation of eosinophils results in various changes in morphology, cell surface characteristics, and functional activities.^{5,15,37} These changes generally are acquired after leaving the circulation, but may be found in circulating eosinophils of patients with allergic disease and

hypereosinophilic syndrome. Activated eosinophils are typically hypodense, have fewer specific granules, and have increased numbers of lipid bodies (Fig. 43.2E) and vesiculotubular structures. Lipid bodies are repositories of arachidonate-containing lipids for oxidative metabolism and synthesis of lipid mediators, and vesiculotubular structures, now termed eosinophil sombrero vesicles (EoSVs), store and transport granule products. Upon activation, EoSVs move to the plasma membrane and fuse with it to rapidly release mediators. In a canine model of asthma, eosinophils found in bronchoalveolar lavage fluid have either a typical polymorphonuclear appearance or the appearance of a globule leukocyte. Other changes noted in human eosinophils are increased primary granules (containing CLCP) and small dense granules. Functionally, cytotoxicity to parasites and mammalian cells is increased, there is enhanced generation of reactive oxygen species, and enzymes, such as acid phosphatase, are activated.

INVOLVEMENT IN HOMEOSTASIS AND THE IMMUNE SYSTEM

In addition to their involvement in inflammation, eosinophils are important to homeostatic and immune

functions, especially mucosal immunity in the GI tract. Under homeostatic conditions, eosinophils traffic into the thymus, mammary gland, uterus, and, most prominently, the GI tract.⁵¹ They are recruited to the thymus during the neonatal period by eotaxin-1 and are associated with thymocyte deletion.⁵⁷ They participate in T cell-mediated immunity through recognition and presentation of microbial, viral, and parasitic antigens, as well as superantigens. The immunologic roles of eosinophils may be related to their location at surfaces in contact with the outside environment, their capacity to respond to particulate antigens (e.g. inhaled, ingested, or granuloma-encapsulated antigens) and their expression of Fc receptors for IgG, IgE, and IgA.⁶³ Eosinophils can also regulate T-cell polarization and proliferation of effector T cells.⁵¹ In several reproductive processes, including development of the post-pubertal mammary gland, onset of estrus cycling, and blastocyst implantation, eosinophils appear to play regulatory roles.^{23,49,51}

TRAFFICKING IN EOSINOPHILIC INFLAMMATION

Trafficking of eosinophils into inflammatory sites is directed by TH₂ and endothelial-derived cytokines, including IL-4, IL-5, and IL-13, adhesion molecules (β 1-, β 2-, and β 7-integrins), chemokines (CCL5, and the eotaxins), and many other molecules, including acidic mammalian chitinase.⁶⁶ Eotaxin-1 was identified in 1994;³⁴ subsequently CCL24 and CCL26 (eotaxins 2 and 3) were discovered, but their sequences are not closely related to CCL11.⁵⁴ In human hypersensitivity disorders, these chemokines are produced by respiratory epithelium and skin fibroblasts; CCL11 appears at the earliest stages, followed by CCL24 and then CCL26.²⁸

The following sequence of events describes the path leading the circulating eosinophil to participate in inflammatory processes:

1. Quiescent eosinophils in the circulation express L-selectin which binds reversibly and with weak affinity to carbohydrate ligands on postcapillary venule endothelium, permitting the eosinophil to roll along the vessel wall.^{37,38}
2. The eosinophil meets a priming stimulus, released from perivascular immune cells, endothelial cells, and the local inflammatory site. Eosinophil primers include IL-3, GM-CSF, IL-5, IgG, and IgA.³⁶ IL-4 mediates ϵ isotype switching and brisk production of IgE.²¹ IL-5 initiates signal transduction in eosinophils resulting in expression of genes for surface receptors, structural and secreted proteins, and transcription factors.^{36,58} Priming leads to adhesion, chemotaxis, degranulation, release of lipid mediators, and oxidative burst.
3. One of the priming functions of IL-5 is to increase the number of priming eosinophils that respond.^{11,47} The primed eosinophils express β 1 (very late activation [VLA]-4), β 2 (Mac-1 and leukocyte function-associated antigen [LFA]-1), and β 7 integrins that mediate

secure adhesion to the endothelial ligands vascular cell-adhesion molecule (VCAM)-1 and intercellular-adhesion molecule (ICAM)-1, induced by IL-1.^{4,5} Although the latter pathway is also used by neutrophils, the VLA-4/VCAM-1 pathway is selective for eosinophils and basophils.⁴ Expression of tissue factor by eosinophils also may play a role in migration from blood.⁴⁶ Adhesion further primes the eosinophil to respond to chemoattractants and lipid mediators.

4. Recruited by various chemotactic factors, the primed eosinophil flattens against the endothelium and migrates between cells into the interstitial space. Depending on the target organ, eosinophils cross the endothelium into tissues by a regulated process involving coordinated interaction between networks involving the chemokine CCL11, eosinophil adhesion molecules (α 4 β 1, α 4 β 7, α m β 2, α L β 2), and adhesion receptors on the endothelium (MAdCAM-1, VCAM-1, and ICAM-1).
5. The eosinophil approaches its final destination, guided by interactions with adhesion molecules in the extracellular matrix (fibronectin, laminin, and fibrinogen) and a concentration gradient of chemoattractants, including C5a, *N*-formyl-methionyl peptides, leukotrienes, platelet-activating factor (PAF), hydroxyeicosatetraenoic acids (HETEs), hydroxyheptadecatrienoic acids (HHTs), monocyte chemoattractant peptide-3 (MCP-3), macrophage inflammatory protein-1a (MIP-1a), eosinophil chemotactic factor of anaphylaxis (ECF-A), histamine, parasite-derived factors, and chemokines like CCL5.^{37,38}
6. Along the way, the eosinophil is activated by cytokines, lipid mediators, chemoattractants, and immunoglobulins to release granule proteins, lipid mediators, and oxygen radicals.
7. Eosinophil survival is enhanced through exposure to inflammatory cytokines, such as IL-5.²⁶
8. Eosinophils may contribute to wound healing, or sclerosis, by secretion of TGF β , procoagulation through activation of factor XII by ECP, and fibrinolysis by activation of plasminogen.^{7,12,59,61}

The predominance of eosinophils in certain disorders, such as parasitic disease and asthma, is likely mediated by several eosinophil-selective molecules, including adhesion molecules, primers, chemoattractants, and anti-apoptotic agents. Finally, a major consequence of chronic eosinophilic inflammation is tissue damage. The injury to airway epithelium and other cells mediated by MBP appears to be reversible, but myocardial fibrosis, perhaps induced by granule proteins or TGF, is permanent in hypereosinophilic syndrome.^{5,22,38}

EFFECTOR FUNCTIONS

Defense Against Helminthic Parasites

Although eosinophils are capable of phagocytosis, helminths are too large for this approach; therefore they

secrete mediators directly onto the target rather than engulfing them. Eosinophils, working in concert with T cell-derived perforins, bind to larvae opsonized with IgG, IgE, or complement, flatten against the integument; release a barrage of basic toxins and hydrolytic enzymes directly onto the target; and migrate under the damaged larval skin.^{6,8,37,43} The oxidative burst in eosinophils may contribute to anti-helminthic action, but the cytotoxic effect of MBP to schistosomula of *Schistosoma mansoni* occurs independently of oxygen metabolites.³⁸ Eosinophils may also contribute to host defense by decreasing the number of infectious agents, dampening immediate pathologic responses, or participating in tissue remodeling and immunomodulation. IL-5 may play a role in the effectiveness of parasite vaccines in mice.⁴¹

The role of eosinophils in helminthic cytotoxicity in vivo remains uncertain. Some human studies suggest a role for eosinophils in preventing reinfection rather than clearing an initial infection.²⁴ Experimental models using transgenic mice that overproduce IL-5 or mice that are deficient in IL-5, eotaxin or the eotaxin receptor have yielded conflicting results about the role of eosinophils and their mediators in helminthic infections. Parasitized mice in which eosinophil and IgE responses were ablated by administering anti-IL-5 antibody had no changes in parasite burden or resistance to infection compared with mice that had intact eosinophil responses.^{33,53} Difficulties in interpreting results arise from use of parasites for which the mouse is not the natural host, from equating murine and human eosinophils, which have functional differences, and from the lack of ability to ascertain that eosinophils are degranulating in response to parasitic infection. After all these years, the beneficial role of eosinophils in control of parasite infection remains to be fully understood.

Effector Cells in Asthma and Allergic Disease

Although eosinophils are thought to play a central role in the pathologic changes that characterize hypersensitivity disorders their definitive contributions are still being determined. Asthma is characterized by inflammation, hyperresponsiveness, mucus secretion, and remodeling with smooth muscle hyperplasia, collagen deposition, neovascularization, and metaplasia of goblet cells.^{3,7} Studies in animals and people suggest an important role for eosinophils in airway remodeling and in acute exacerbations in asthma, but many uncertainties remain.

Eosinophils promote damage and dysfunction through elaboration of proinflammatory mediators, such as leukotriene C₄, PAF, and eicosanoids, and release of granule proteins.⁶⁷ Eosinophils are recruited into tissues by cytokines. In an amplification loop, eosinophils themselves may recruit CD4⁺ cells into tissues by releasing IL-16. IgE-mediated mast cell degranulation, induced upon re-exposure to allergens and para-

site antigens, also plays a role in eosinophil recruitment in hypersensitivity disorders.³⁷ Eosinophil granule products, reactive oxygen species, and lipid mediators cause damage to airway mucosa and nerves and also cause bronchoconstriction. Major basic protein in particular is toxic to airway epithelium and triggers degranulation of mast cells and basophils.⁵¹ Eosinophils from patients with asthma are hyperadhesive and have increased collagen receptor expression. TGF- β from eosinophils may play an important role in airway remodeling. Leukotrienes secreted by eosinophils mediate vascular permeability, secretion of mucus, and constriction of smooth muscle. In addition, cytokines involved in recruitment also enhance effector functions and prolong survival of eosinophils by delaying apoptosis.

Modulation of Inflammation

Eosinophils suppress immediate hypersensitivity reactions.³⁷ Specifically, eosinophils phagocytose immune complexes, peroxidize mast cell granule debris, and release histaminase to neutralize histamine, phospholipase B to inactivate mast cell PAF, MBP to inactivate mast cell heparin, plasminogen to reduce local thrombin activity, aryl-sulfatase B and hypochlorous acid to inactivate leukotrienes, prostaglandin E₁ (PGE₁) and PGE₂ to inhibit degranulation of basophils and mast cells, and lysophospholipase to prevent generation of arachidonic acid metabolites.

Phagocytosis

Eosinophils have the capacity to phagocytose immune complexes, antibody-coated RBCs, mast cell granules, inert particles, yeast, and bacteria, including *Mycoplasma*. They are less efficient at killing bacteria than neutrophils despite having higher levels of peroxidase activity, oxidative responses, and H₂O₂ production.^{14,32} Eosinophils lack several bactericidal substances and have a lower density of complement receptors than neutrophils.

Antitumor Effect

Eosinophils and their products are cytotoxic to or induce apoptosis of tumor cells in vitro,³³ and antitumor roles for IL-5 and eosinophils are suggested in murine models of increased expression of IL-5 and eotaxin deficiency, respectively. In some neoplasms, local infiltration by eosinophils in the absence of peripheral eosinophilia improves prognosis.⁴⁰ In human colorectal carcinomas, improved prognosis associated with eosinophil infiltration was independent of staging, vascularization, p53 expression, and histologic grade.¹⁹ However, in other studies eosinophilic infiltrates in tumors have been associated with a poorer prognosis or had no impact on prognosis.^{1,39}

MECHANISMS OF EOSINOPHIL EFFECTOR FUNCTION

Release of Granule Proteins

Granule products are released through exocytosis, piecemeal degranulation, or cell lysis.^{44,52} In piecemeal degranulation, granule products are carried to the plasma membrane by transport vesicles with resultant disassembling (Fig. 43.2B–D) and emptying of granules. Unique to eosinophils is a dynamic system of vesicular compartments, termed eosinophil sombrero vesicles (EoSVs), generated by the specific granules themselves. Using electron tomography, which permits three-dimensional imaging of subcellular structures, EoSVs are demonstrated to be highly pleomorphic curved tubular structures that may be round, C-shaped, or elongate (Fig. 43.2C,D). They can change shape to accommodate transport of membrane-bound proteins over long distances in the cell and store certain proteins for quick release upon stimulation.⁴⁴ EoSV “dock” and then fuse with the plasma membrane, releasing proteins. The docking complex consists of soluble N-ethylmaleimide-sensitive factor attachment receptors (SNARE) located on the plasma membrane and the vesicle. When eosinophils are activated, EoSVs move to the plasma membrane to permit rapid release of mediators. Piecemeal degranulation is selective and tightly regulated so that only specific proteins are released after receiving a distinctive stimulus. For example, IgE induces release of ECP and IgA binding can cause release of ECP and EPO, whereas IgG binding results in release of ECP and EDN.^{35,37,43} Equine eosinophils degranulate through a complex process of compound exocytosis and cumulative fusion in which multiple granules fuse with one another after fusion of one granule with the plasma membrane, resulting in a degranulation sac and focal release of granule proteins.⁵²

Release of Lipid Mediators

Eosinophils not only respond to PAF, they produce and secrete it on activation. Platelet-activating factor enhances many eosinophil activities including adherence to endothelium, chemotaxis, binding of IgE, production and release of oxygen radicals, release of granule proteins, and synthesis of prostanoids. Other lipid mediators released by eosinophils include leukotrienes (e.g. LTB₄ and LTC₄), thromboxane A₂, and prostaglandins.^{37,38} With the exception of PGE₂, which may down-regulate eosinophil effector functions, lipid mediators contribute to host dysfunction, such as bronchial hyperreactivity.³⁸

Cytokine Release

Eosinophils exhibit paracrine regulation of other inflammatory cells, endothelial cells, and fibroblasts through release of IL-1, IL-2, TNF α , TNF β , IL-4, IL-5, IL-6, IL-8,

IL-10, IL-12, IL-13, IL-16, IL-18, TGF α/β , eotaxin, and CCL5. Autocrine control results from elaboration of IL-3, IL-5, and GM-CSF by eosinophils.^{5,63}

MECHANISMS OF EOSINOPHILIA AND EOSINOPENIA

Factors influencing the number of eosinophils in the circulation include: diurnal variation related to cortisol levels, age, exercise, and exposure to environmental stimuli. Eosinophils are enumerated in several ways. The least accurate is the differential white blood cell (WBC) count performed on a stained blood film, but this method is useful in most situations. Eosinophil counting chambers also can be used, but automated methods that stain peroxidase provide the most accurate counts. Eosinophil peroxidase is not detected by this method in all animal species, especially cats. The reference interval for eosinophils varies among species and geographic regions. Eosinophilia is not always present in the face of eosinophilic inflammation in tissues where eosinophil survival is increased. Specific causes of eosinophilia and eosinopenia are discussed in Chapters 46–50.

Eosinophilia

The pathway to eosinophilia in most causes of eosinophilic inflammation is the elaboration of eosinophilopoietic factors, principally IL-5, by T cells sensitized by parasite antigens or allergens.²⁵ IL-5 derived from mast cells and eosinophils probably plays a local role in priming eosinophils, enhancing their function, and prolonging their survival.⁶⁴ Eosinophilia associated with parasitism is more likely to occur when parasites are located in tissues rather than in the intestinal lumen. In eosinophilic bronchopneumopathy (EBP), previously termed pulmonary infiltrates with eosinophilia (PIE), eosinophilic infiltrates are found in the bronchial mucosa and interstitium,^{9,10} and hypersensitivity in this syndrome may be initiated by heartworm infection, migration of larval parasites, exogenous proteins, and antigens associated with chronic infections. Chronic eosinophilia is associated with inflammatory disorders of mast cell-rich organs, namely skin, lung, GI tract, and uterus. Other conditions include eosinophilic granuloma complex and eosinophilic inflammatory bowel disease in cats and eosinophilic myositis, eosinophilic panosteitis, and eosinophilic gastroenteritis in dogs. Paraneoplastic eosinophilia is caused by a variety of tumors, such as lymphoma, mast cell tumor, and solid tumors, in which IL-5 and other cytokines are elaborated.¹⁸ Eosinophilic leukemia is a rare disease, and the diagnosis depends on ruling out other causes and measuring serum IgE levels (see Chapter 63). In hypereosinophilic syndrome (HES), characterized by persistent eosinophilia of unknown origin, increased survival of eosinophils in circulation, tissue infiltrates, and organ dysfunction, a proposed mechanism of eosinophilia is

clonal expansion of T cells generating eosinophilopoietic factors.⁶² Increased levels of IL-5 in some of these patients may override the apoptotic effects of corticosteroids, resulting in steroid resistance. In a subset of people with HES, constitutive expression of tyrosine kinases leads to production of eosinophils independent of growth factors.⁵¹ Eosinophilia is sometimes recognized in animals with hypoadrenocorticism owing to decreased or absent cortisol.

Eosinopenia

Detection of low numbers of eosinophils, especially on a routine differential WBC count, has limited significance, and some healthy animals have no eosinophils detected in a complete blood count. However, there are several known associations with eosinopenia, although the underlying mechanisms are unclear. For example, corticosteroids cause eosinopenia, perhaps by neutralization of circulating histamine and inhibition of mast cell degranulation. The lympholytic effect of corticosteroids may attract eosinophils into lymphoid tissues after release of cytokines; alternatively, production of cytokines promoting growth and function of eosinophils may be diminished. Corticosteroids also inhibit cytokine-dependent eosinophil survival. Thus, eosinopenia may occur in some animals with hyperadrenocorticism or following exogenous administration of corticosteroids. Other inducers of eosinopenia include catecholamines. This may occur through a β -adrenergic effect, as the effect is eliminated by β -blockers. Eosinopenia may be present in acute inflammation or infection, in part owing to release of corticosteroids and catecholamines.

THERAPEUTIC APPROACHES

Understanding the molecular and cellular mechanisms of eosinophilic inflammation has opened the door to novel therapeutic interventions that may transform medical management of patients with eosinophilic inflammation.^{5,28,38,51} Glucocorticoids have been the therapeutic mainstay and act by destabilizing eotaxin mRNA, suppressing transcription of IL-4, IL-5, and eotaxin genes, and inhibiting survival of eosinophils. However, not all patients respond or maintain a response to glucocorticoids, possibly owing to loss of receptors or alterations in regulatory proteins. Other approaches include reduction of eosinophil production by myelosuppressive drugs, such as hydroxyurea, inhibition of eosinophil degranulation and effector functions by IFN- α , and interference with transcription of cytokines, such as IL-5 and GM-CSF by cyclosporin A. Novel approaches include anti-IL-5 antibodies; antagonists to leukotrienes, such as cysteinyl leukotrienes, or their receptors; tyrosine kinase inhibitors, such as imatinib, that may be effective in patients with HES caused by constitutive production of tyrosine kinases; lidocaine, recently demonstrated to shorten eosinophil survival; third generation anti-histamines; blockers of

eosinophil adhesion; inhibitors of the eotaxin/CCR3 pathway; anti-human IL-13 antibody; and induction of eosinophil apoptosis by antibody cross-linking of Siglec-8.

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Basophils, Mast Cells, and Their Disorders

LISA M. POHLMAN

Production and Tissue Distribution

Basophils
Mast Cells

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Basophils
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Acronyms and Abbreviations

AP-1, activation protein-1; bFGF, basic fibroblast growth factor; CD, cluster of differentiation; C3biR, inhibitory receptor for C3b; CTAP-III, connective tissue-activating protein III; ELAM, endothelial-leukocyte adhesion molecule; ERK, extracellular receptor-activated kinase; Fc, fragment crystallizable; FIC, fibroblast induced cytokine; GM-CSF, granulocyte-macrophage colony stimulating factor; ICAM, intercellular adhesion molecule; H&E, hematoxylin & eosin IFN- γ , interferon-gamma; Ig, immunoglobulin; IgA, immunoglobulin A; IgE, immunoglobulin E; IgG, immunoglobulin G; LFA, lymphocyte function-associated antigen; IGF, insulin-like growth factor; IL, interleukin; IFN, interferon; LT, leukotriene; LTC₄, leukotriene C₄; LTD₄, leukotriene D₄; LTE₄, leukotriene E₄; MAC-1, macrophage-1 antigen; MAP, mitogen-activated protein; MCP-1, monocyte chemotactic protein 1; MIP-1a, macrophage inflammatory protein-1a; MC_T, mast cell subset with tryptase; MC_{TC}, mast cell subset with tryptase and chymase; NGF, nerve growth factor; NK, natural killer; NFAT, nuclear factor of activated T-cells; NF- κ B, nuclear factor κ B; PAF, platelet-activating factor; PGD₂, prostaglandin D₂; PECAM, platelet/endothelial cell adhesion molecule; Pgp-1, phosphoglycoprotein-1; PLA₂, phospholipase A₂; RANTES, regulated on activation normal T cell expressed and secreted; SCF, stem cell factor; TGF- β , transforming growth factor beta; Th1, T-helper lymphocyte type 1; Th2, T-helper lymphocyte type 2; TLR, toll-like receptor; TNF, tumor necrosis factor; TxB₂, thromboxane B₂; VCAM, vascular cell adhesion molecule; VEGF, vascular endothelial growth factor; VLA, very late antigen; VNR, vitronectin receptor.

Because basophils and mast cells are alike in chemical composition and function, they are typically referred to as sister cells. However, these cells function independently^{30,33} and are immunophenotypically distinct from each other as well as from other hematopoietic cells.⁹ Also in the few reported cases of defective basopoiesis or basophil function, patients had no concurrent mast cell defect.¹⁵

PRODUCTION AND TISSUE DISTRIBUTION

Basophils

Basophils, are terminally differentiated granulocytes that originate from CD34⁺, Fcg, c-kit⁻ cells in the bone marrow (see Chapter 7).¹⁹ They are the least numerous blood granulocyte in domestic mammals constituting

about 0.5% of blood leukocytes in health. Basophilic myelocytes can be identified in the bone marrow using routine hematologic stains. Normally, the process of maturation takes about 2.5 days and is completed in the bone marrow.¹² Once in the peripheral blood, they circulate briefly (half-life is approximately 6 hours) but may live as long as 2 weeks in the tissue.¹⁷ Basophils may be recruited into tissue during an immunologic or inflammatory response. Once there, basophils retain their unique morphologic features, but are not routinely identified in hematoxylin and eosin-stained tissue sections.

IL-3 is the major basophil growth factor and cytokine effector of basophil function. It promotes the production, differentiation and survival of basophils *in vitro*, and results in upregulation of essentially all basophil responses.²⁷ IL-3 is especially important for bone marrow basophil hyperplasia and blood basophilia in conjunction with Th2 immunologic responses. Other growth factors that contribute to basophil production and differentiation are GM-CSF, IL-5, SCF, IL-4 and other cytokines (Table 44.1). These cytokines generally either induce mediator release from basophils or augment release of mediators stimulated by other agonists. In addition to directing basophils to inflammatory sites, chemotactic cytokines (Table 44.1) also produce or enhance basophil release functions.³ Further, TGF- β , in the presence of IL-3, curbs eosinophil differentiation and augments basophil production, providing evidence for reciprocal control of the maturation of pluripotent progenitor cells into basophils or eosinophils.²⁶

Basophils accumulate at sites of inflammation. To do so, basophils must emigrate from the blood into and through tissues in response to chemotactic agents (Table 44.1).^{3,24,31} After stimulation, basophils release cytokines, including TNF- α and IL-4, that increase expression of adhesion molecules on endothelial cells, thereby initiating the first step of emigration. Rolling of basophils along endothelium is mediated by basophil selectins, which act to tether the cells loosely to their carbohydrate counter ligands on endothelial cells. Adherence of basophils to the vascular endothelium involves ICAM-1, ELAM-1 and VCAM-1 that are expressed on the cell surface of activated endothelial cells. Basophils express integrins as receptors for these molecules and these integrins and members of the immunoglobulin superfamily then act to establish firm adhesion. Trans-endothelial migration and movement through the extracellular matrix also are mediated by similar mechanisms.

Mast Cells

Mast cells originate in the bone marrow from agranular, CD34+, pluripotent, hematopoietic stem cells, that differentiate into a separate and distinct lineage from monocytes/macrophages and from granulocytic precursors.^{2,4,8,13,18} Researchers have identified the CD34+, c-kit+, and CD13- precursor cells that, in the presence

of specific growth factors, will differentiate into mast cells. Mast cell survival, growth, and differentiation are promoted by SCF (a mast cell hemopoietin that is produced by endothelial cells, epithelial cells, fibroblasts and other cell lines in culture) and inhibited by GM-CSF, IFN- γ and TGF- β .

Normal interactions between SCF and c-kit and the resulting molecular signaling that follows are critical for the development of normal mast cells.²¹ Mast cells deprived of SCF undergo apoptosis, whereas mutations in c-kit that result in increased expression of the c-kit proto-oncogene can cause mastocytosis and mast cell neoplasia (see Chapter 68).²¹

Primed by SCF and other growth factors (Table 44.1), committed mast cell precursors leave the bone marrow early in their development, and in normal mammals, circulate as agranular, c-kit+, CD34+, CD13+ mononuclear cells.¹² Mast cell precursors eventually migrate into connective tissue or the lamina propria of the mucosa to proliferate, differentiate, mature, and accumulate their characteristic cytoplasmic granules under the influence of a variety of interleukins such as IL-3, IL-4, IL-9 and IL-10.^{18,29}

BIOLOGIC FUNCTIONS

Basophils

Basophils have an important role in the late-phase of the type-1 (immediate-type) hypersensitivity reaction,^{9,20} as well as during the early phase of the cell-mediated delayed-type hypersensitivity response. Circulating basophils enter tissues at sites of inflammation during the late phase of the IgE-mediated immediate hypersensitivity reaction where, like mast cells, they have deleterious, rather than protective effects.²⁶ Their important role as mediators of an allergic response is illustrated in genetically engineered mast cell deficient mice that are capable of mounting an IgE-mediated anaphylactic response.¹⁶ Extensive evidence exists supporting the role of basophils in allergic conditions such as allergic rhinitis, hives, or urticaria, asthma, allergic conjunctivitis, allergic gastritis, and drug-induced or insect-bite-induced anaphylaxis.^{7,16}

Even though the phagocytic potential of basophils is minimal, one of their protective functions involves the defense against helminths.²⁶ This is achieved by the stimulation of T cells to produce a Th2 response. In fact, in the absence of basophils, Th2 cytokine production by T cells is minimal or absent.

Basophils have been reported to have a critical function in the development of IgE-mediated chronic allergic inflammation, where they are necessary for recruitment of inflammatory cells including neutrophils and eosinophils.²⁵ Basophils may also antagonize or promote hemostasis by means of the anticoagulant actions of secreted heparin and the procoagulant effects of kallikrein. They may promote lipolysis by activation of lipoprotein lipase through secretion of heparin.

TABLE 44.1 Major Properties of Basophils and Mast Cells^a

Characteristic	Basophils	Mast Cells
Pluripotential stem cell	CD34+	CD34+
Origin of precursor cells	Bone marrow	Bone marrow
Site of differentiation and maturation	Bone marrow	Connective tissue (a few in marrow)
Nucleus	Long ribbon-like, variably segmented	Round to oval
Recruitment of mature cells into tissue	Yes (in inflammation)	No
Mature cells normally present in connective tissue	No	Yes (located perivascularly, around nerves and beneath the epithelium)
Circulation of mature cell in blood	Yes, but low numbers	Rare
Proliferative ability of mature cells	None reported	Yes
Lifespan	Days (similar to other granulocytes)	Weeks to months
Major growth factors mediating differentiation and proliferation	IL-3	SCF, IL-3, IL-6, CCL11, CCL24, CCL26, NGF
Surface structures		
TLRs	TLR1 (mouse), TLR2 and TLR4 (human and mouse), TLR6 (mouse)	TLR1, TLR2, TLR3, TLR4, TLR6, TLR7, TLR9
Immunoglobulin receptors	FcεRI, FcεRII (CDw32)	FcεRI, FcεRII
Expression of FcγRI	High	High
Cytokine/growth factor receptors	IL-1R, IL-2R (CD25), IL-3R, IL-4R, and IL-8R, chemokine and interferon receptors, +/- SCFR (c-kit) (CD117)	SCFR (c-kit) (CD117)
Cell adhesion structures	P24 (CD9), LFA-1 chain (CD11a), C3biR, Mac-1 (CD11b), LFA-1 chain (CD18 and CD29), PECAM (CD31), leukosialin (CD43), Pgp-1 (CD44), VLA-4 (CD49d), VLA-5 (CD49e), ICAM-3 (CD50), ICAM-1 (CD54), LFA-3 (CD58), ICAM-2 (CD102)	CD9, CD29, CD43, CD44, CD49d, CD49e, CD50, VNR, CD51, CD54, CD58, LFA-1, chain, CD61, CD102
Major cell products		
Stored preformed in cytoplasmic granules		
Biogenic amines	Histamine, adenosine, serotonin (rats and mice)	Histamine, serotonin (rats and mice)
Enzymes	Neutral proteases with bradykinin-generating activity, elastase, β-glucuronidase, cathepsin G-like enzyme	Tryptase, chymase (M _{TC}), carboxypeptidase (M _{TC}), cathepsin G (M _{TC}), acid hydrolases, phospholipase A ₂ , aminopeptidase, hexosaminidase, chitinases
Proteoglycans	Chondroitin sulfate, heparin, dermatan sulfate	Heparin and/or chondroitin sulfate
Other	Major basic protein	
Mediators synthesized de novo		
Arachidonic acid products	LTC ₄	PGD ₂ , LTC ₄ , LTB ₄ , LTE ₄ , TxB ₂ , PGE ₂
Cytokines	IL-4, IL-8, IL-13, TNF-α, CCL3	SCF, TNF-α, IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-16, GM-CSF, MCP-1, CCL3, CCL4, bFGF, CCL4, endothelin, lympholactin, VEGF, NGF
Other	PAF	PAF
Agonists		
Cytokines	IL-1, IL-2, IL-3, IL-4, IL-5, IL-8, CD40 ligand, GM-CSF, IFNγ, IGF-I and II, TGF-β, NGF, chemokines	IL-3, IL-4, IL-9, IL-10, NGF, GM-CSF, SCF, endothelins
Chemokines	CCL1, CCL3, CCL4, CCL6, CCL7, CCL24, CCL26	IL-8, CCL2, CCL3, CCL5
Chemotaxins	IL-3, GM-CSF, IL-5, IL-8, PAF, C5a and chemokines	
Complement proteins	C1, C3, C4, C5a	C3a, C5a
Metachromatic staining with basic dyes		
At acid pH	Yes	Yes
At neutral pH	No	Yes
Cytochemical staining		
Chloracetate activity	Negative usually (except cats)	Positive
Tryptase activity	Negative	Positive

^aSpecies variations exist.

Mast Cells

Mast cells function to promote hypersensitivity reactions characterized by vasodilation, increased vascular permeability, bronchoconstriction, mucus secretion, and nerve stimulation; they modulate the immune response by stimulating T cells; they play a role in host defense against tissue parasites; and they promote acute and chronic inflammatory responses by stimulating leukocyte migration, endothelial-leukocyte adhesion, angiogenesis, fibrin deposition, fibroblast proliferation, and fibrosis.²³

Mast cells have long been recognized as tissue cells with critical roles in type 1 hypersensitivity reactions. More recently, mast cells have been established as tissue leukocytes with key functions in host defense, inflammation, tissue remodeling and fibrosis, as well as coagulation.²⁸ Research is currently being conducted on the role of mast cells in asthma, atherosclerosis, myocardial infarction, human immunodeficiency virus, cocaine abuse, fibrotic disorders, and rheumatological disorders (Table 44.1).

BINDING OF IgE TO BASOPHILS AND MAST CELLS

Both basophils and mast cells express the $\alpha\beta\gamma_2$ form of the high-affinity, surface Fc receptor for IgE, known as Fc ϵ RI. Fc ϵ RI is specific for ϵ -heavy-chains. In healthy people and animals, these IgE molecules vary significantly in antigen specificity.³² Conversely, in atopic patients, many of the IgE molecules that are bound to mast cells and basophils are specific for one or only a few different antigens.³² This lack of IgE variability, upon exposure to the specific antigens, results in cross-linking of the Fc receptors, which results in rapid cell activation and degranulation. Activation and degranulation of tissue mast cells then triggers an immediate hypersensitivity reaction (Fig. 44.1), as well as sequential recruitment of basophils to tissue sites, thereby expanding the spectrum of the inflammatory process.⁵

ACTIVATION, DEGRANULATION, AND PRODUCTION OF MEDIATORS BY BASOPHILS AND MAST CELLS

There are numerous initiators of mast cell and basophil activation such as cross-linking of IgE by antigen, cytokines, chemokines, chemical agents (e.g. radiocontrast solutions), physical stimuli (e.g. cold), peptides (e.g. neutrophil lysosomal proteins, insect and animal venoms, bacteria, and bacterial products), viruses, calcium ionophors, narcotics, and muscle relaxants. IgE-mediated activation and degranulation is the most well understood of these phenomena so it will be the focus of the discussion.

When antigen binds to two adjacent IgE molecules, it induces aggregation of the IgE molecules and associated Fc ϵ RI receptors (Fig. 44.1).¹⁸ This event initiates a

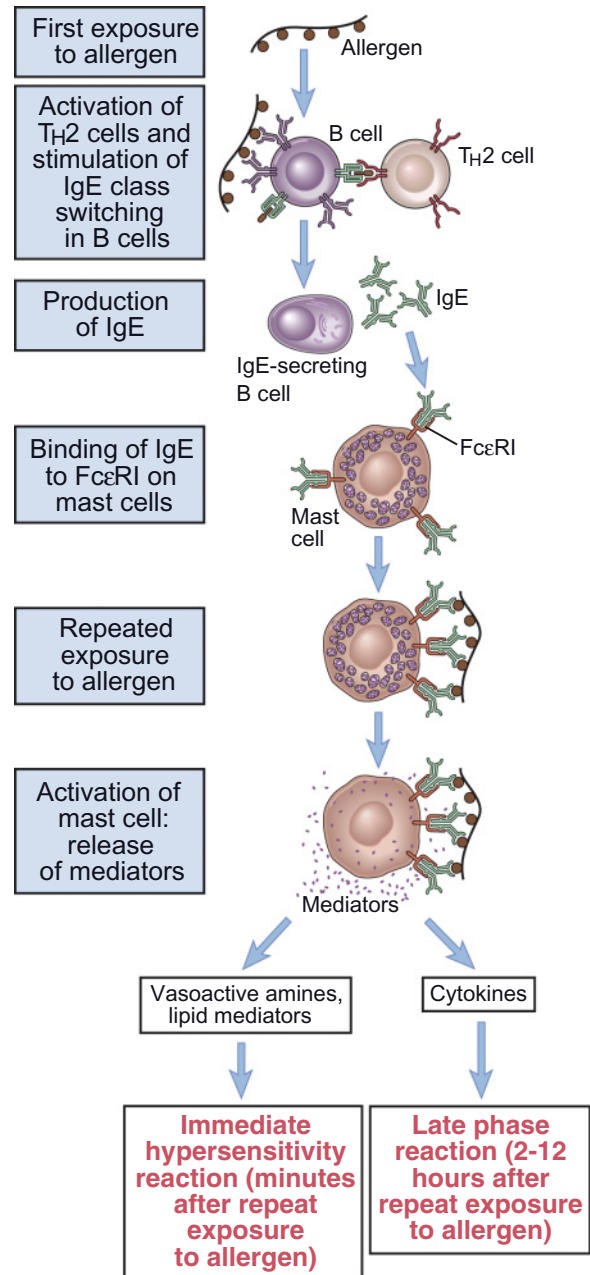


FIGURE 44.1 Order of events in type 1 (immediate-type) hypersensitivity. Type 1 hypersensitivity diseases begin with exposure to an allergen. On first exposure, the allergen stimulates a Th2-type response and production of IgE. IgE binds to Fc ϵ RI receptors on mast cells and results in sensitization. With subsequent exposure to the allergen mast cells are activated to secrete mediators that are responsible for the pathologic effects of type 1 hypersensitivity. (Adapted with permission from Abbas AK, Lichtman AH, Pillai S, Immediate hypersensitivity, Chapter 19, In: Cellular and Molecular Immunology, 6th ed. Philadelphia: Saunders, 2007. ©Elsevier.)

signaling cascade resulting in three types of biological responses: (1) secretion of the preformed contents of the granules (including histamine), (2) synthesis and secretion of lipid mediators, and (3) synthesis and secretion of cytokines (Table 44.1), all of which work together to initiate a local inflammatory response.¹

Histamine is a short-lived vasoactive amine that causes an immediate increase in local blood flow and vessel permeability. Enzymes, such as chymase, trypsin, and serine esterases, activate matrix metalloproteases. Matrix metalloproteases in turn, cause tissue destruction by breaking down tissue matrix proteins.

Synthesis and secretion of lipid mediators is induced via the activation of the cytoplasmic enzyme phospholipase A₂ (PLA₂). This is activated by two signals: (1) increased cytoplasmic calcium and (2) phosphorylation catalyzed by mitogen-activated protein (MAP) kinases such as extracellular-regulated kinase (ERK). Activation of PLA₂ results in hydrolysis of mast cell membrane phospholipids and the production of arachidonic acid, which is converted by cyclooxygenase or lipoxygenase to subsequently produce prostaglandin and leukotrienes respectively.² Protein kinases also act on genes coding for cyclooxygenases and lipoxygenases to promote transcription and increased expression.¹ The major arachidonic acid-derived mediators produced by mast cells are PGD₂, LTC₄, LTD₄ and LTE₄. Basophils produce predominantly LTC₄.¹ PAF causes bronchoconstriction, retraction of endothelial cells, and relaxation of vascular smooth muscle.¹

IgE-mediated synthesis and secretion of cytokines occurs as a result of activation of protein kinases, which

in turn results in the induction of gene transcription factors such as NFAT, and NF- κ B, as well as activation of AP-1. These transcription factors stimulate transcription of IL-4, IL-5, IL-6, IL-13, and TNF, among others.² Both mast cells and basophils also express Fc receptors for IgG1 that can be activated by cross-linking bound IgG1 (Fig. 44.2). IgE is, however, the major antibody involved in mast cell and basophil activation.¹

Mast cell degranulation is also controlled by surface catecholamine receptors (α - and β -adrenoceptors) that have opposing effects. Mast cell degranulation is augmented by molecules that stimulate α -receptors (e.g. norepinephrin and phenylephrin) or block β -receptors (e.g. propranolol). Conversely, mast cell degranulation is inhibited by molecules that stimulate β -receptors or block α -receptors. β -Stimulants are widely used in the treatment of allergies and include isoproterenol, epinephrine and salbutamol. β -Blockade, resulting in increased mast cell degranulation and tissue inflammation, can be caused by some respiratory pathogens such as *Bordetella pertussis*, or *Haemophilus influenzae*.³² Animals with these infections also have an increased predisposition to development of respiratory allergies.³²

Activation of mast cells and basophils is regulated by a variety of inhibitory receptors. Of these, one of the most important is Fc γ RIIb (Fig. 44.2). Experiments in

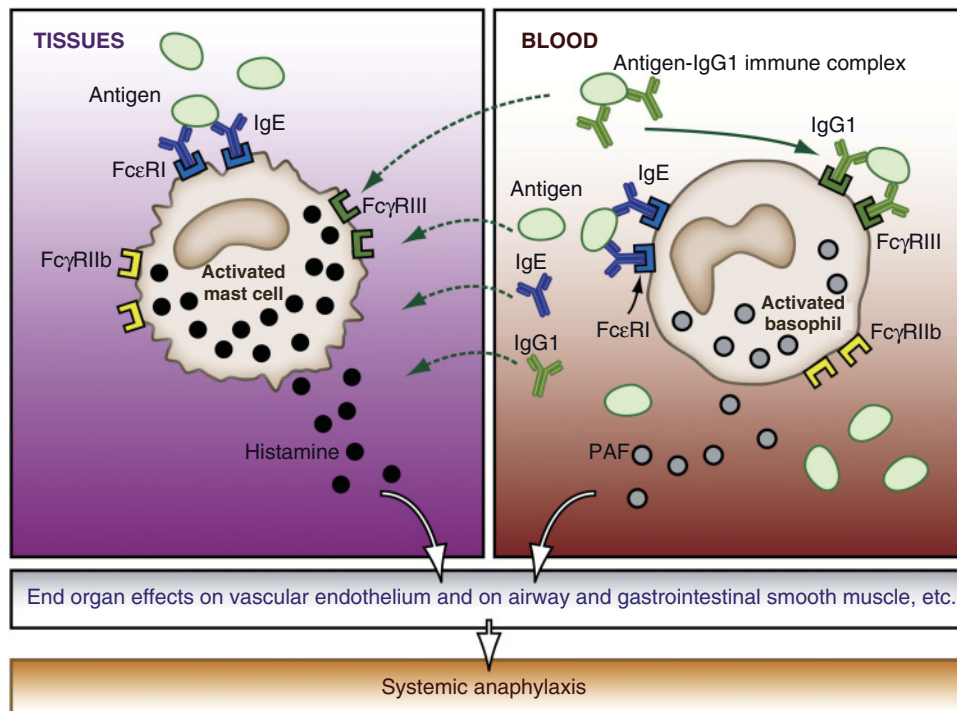


FIGURE 44.2 Contributions of mast cells and basophils to systemic anaphylaxis. Intravenous injection of antigen in sensitized mice is recognized by basophils that have bound antigen-specific IgG1 antibodies. Antigen and circulating IgE and IgG1 complexes can gain access to mast cells by diffusion through vascular endothelium and tissues especially in the face of increased vascular permeability. Antigen-specific IgE and IgG1 are also produced in tissues. When surface Fc ϵ RI is aggregated by the binding of Fc ϵ RI-bound IgE to bi- or multivalent antigen, basophils and mast cells are activated to secrete mediators. Both cell types also can be activated when surface Fc γ RIII binds IgG1 immune complexes and specific antigen. If antigen bound to IgG1 antibodies colligates Fc ϵ RIII or Fc ϵ RI and the inhibitory receptor, Fc γ RIIb, mast cell and basophil activation is repressed. Basophil-derived PAF is a major mediator of IgG1-dependent systemic anaphylaxis. (Reproduced from Galli SJ, Franco CB, Basophils are back! *Immunity* 2008;28:495–497, with permission. ©Elsevier.)

mice show that Fc γ RIIb can regulate mast cell activation *in vivo*.¹ Pharmacologic inhibitors of mast cell and basophil activation include corticosteroids, cyclosporine A, and theophylline.¹⁷

MAJOR PRODUCTS OF MAST CELLS AND BASOPHILS

The primary substances stored in mast cell and basophil granules include biogenic amines, enzymes, proteoglycans, and, in basophils, major basic protein (Table 44.1).⁹ The secondary products that are synthesized *de novo* subsequent to activation include lipid-derived mediators and cytokines. IL-4, that recruits eosinophils and helps drive immunoglobulin class switching and IgE production, is the major cytokine synthesized by basophils. Degranulated mast cells and basophils will regenerate their granules.³²

ROLE OF MAST CELLS AND BASOPHILS IN ALLERGIC DISEASE

Mast cells and basophils are the central components and major effector cells of immediate hypersensitivity reactions and allergic diseases. The cytoplasmic granules of both cells contain the major mediators of hypersensitivity reactions (Table 44.1).¹ Type 1 hypersensitivity is a form of inflammation that primarily involves cross-linking of Fc ϵ RI by antigen on mast cells and basophils, which results in abrupt release of inflammatory mediators (Fig. 44.2). The clinical and pathologic manifestations depend on the tissue location and the number of effector cells responding. The number of effector cells responding depends on: (1) the degree to which the animal has been previously sensitized, (2) the quantity of antigen to which the animal is exposed, (3) the route of antigen administration, and (4) the chronicity of the resulting inflammatory process.^{1,32}

Exposure to environmental antigens is a part of daily life. There is no obvious clinical consequence in most animals because they respond by producing IgG or IgA antibodies. However, some animals produce excessive amounts of IgE antibody, which is the result of an exaggerated Th2 response (Fig. 44.1).³² Basophils are believed to be key players in this exaggerated Th2 response because they alter lymphocyte responses and promote the Th2 response by providing the initial IL-4 required to activate the CD4⁺ T cells. *In vitro* studies have also shown that basophils induce switching of isotype to IgE.

INTERACTIONS OF BASOPHILS AND MAST CELLS WITH EOSINOPHILS

Mast cells, basophils, and eosinophils frequently interact in inflammation, with each having both protective and pathologic effects.³² Mast cells produce IL-5 and CCL11, CCL24 and CCL26 that stimulate the release of

eosinophils from the bone marrow.³² Also, molecules such as CCL11, CCL24 and CCL26, histamine, LTB₄, 5-hydroxytryptamine, and PAF attract eosinophils to the sites of inflammation.³² Basophil influx occurs in the late-phase of the allergic response. Eosinophil degranulation releases major basic protein and PAF causing more mast cells and basophils to degranulate. This effect is augmented by mast cells, which further synthesize and secrete IL-3, IL-5 and GM-CSF, thereby enhancing eosinophil and basophil growth, differentiation, and activation.³²

MAST CELLS AND BASOPHILS IN INFLAMMATION AND HOST DEFENSE

Most of our knowledge of mast cell and basophil-mediated responses comes from the analysis of hypersensitivity diseases. Even though mast cells and basophils have traditionally been thought of in terms of their association with the allergic response, studies indicate that these cells also provide protective functions with roles in innate immunity, host defense and inflammation.¹ Hosts must be able to mobilize defenses and direct them to the site(s) of pathogen growth when organisms cross epithelial barriers and attempt to establish foci of infection. Activation of mast cells, which are sentinel cells, is one mechanism by which this is achieved.³²

The location of mast cells near body surfaces allows them to recruit both specific and nonspecific effector elements to sites where infectious agents are most likely to enter the body.³² In response to microbial invasion, tissue damage, bacteria and bacterial products, and inflammatory mediators, including defensins, neuropeptides, adenosine, and endothelins, mast cells and basophils release potent inflammatory mediators, such as histamine, proteases, chemotactic factors, cytokines and metabolites of arachidonic acid.²⁹ Collectively, these act on the vasculature, smooth muscle, connective tissue, and mucous glands, and attract inflammatory cells to the lesion. This release is carefully regulated to ensure that the severity of inflammation is appropriate to the body's immediate needs. Therefore, in a healthy inflammatory response, release of inflammatory mediators occurs relatively slowly (Table 44.1).

Activation of mast cells and basophils results in increased flow of lymph from sites of antigen deposition to regional lymph nodes, where naïve lymphocytes are first activated. IgE-mediated mast cell and basophil responses are critical in defense against parasite infection.¹ IgE-dependent mast cell activation in the gastrointestinal tract and lungs has also been shown to promote the physical expulsion of parasites by initiating an outpouring of mucus and triggering muscular contractions, thereby increasing peristalsis in the gut.¹ IgE-mediated mast cell and basophil responses are also protective against blood-sucking ticks. On first exposure, skin at the site of a tick bite contains degranulated mast cells, basophils, and eosinophils. However, with subsequent tick-feedings, resistance to the tick bite

typically develops. In dogs and cats, heartworm infection frequently induces an increase in basophils both in the tissues and in the blood.

Mast cells also have an important protective role in the innate immune response to invading bacteria; the mediators they release during these responses are critical for clearing the infection. Moreover, studies in mice indicate that during an acute bacterial infection, mast cells are activated by IgE-independent mechanisms.¹

Mast cells are present in significant numbers in heart tissue of many species including amphibians, mice, rats, dogs, and humans. They release many inflammatory mediators at the site of ischemia that are important in both the proinflammatory phase and the proliferative/fibrosis phase of infarct healing.¹⁴

LABORATORY EVALUATION OF BASOPHILS

Microscopic Appearance

When seen on blood films, normal mammalian basophils are larger than neutrophils, with a diameter similar to that of eosinophils. Like other granulocytes in domestic species, the nuclei of basophils are mildly segmented and have mostly condensed chromatin. The nuclei of canine and feline basophils frequently extend to the margins of the cell and may be described as long and ribbon-like with twisting and folding, but with less segmentation and chromatin clumping than mature neutrophils, and fewer nuclear filaments. The cytoplasmic granules of mammalian basophils are variably sized, round to oval and typically stain metachromatically with basic dyes such as Alcian blue and toluidine blue at low pH. The amount of granulation varies between species (see Section IX).

Basophil Histamine Release Assays

Basophils contain most of the histamine in blood. Therefore, in an attempt to assess hypersensitivity states in several species, including horses and sheep, assays for basophil histamine release have been developed.²²

Basophil Counts

Automated methods for counting basophils are usually based on the resistance of human basophils to lyse in fluids that lyse other leukocytes. This method does not appear to work for the dog and cat. For example, The Technicon H1®, Advia 2120®, Sysmex XT 2000 iV®, and Lasercyte® all failed to accurately detect basophils when basophils were present in large numbers in canine cases, (Dr. Harold Tvedten, personal communication).³³ The Sysmex XT 2000 iV® did not detect basophilia in feline cases, but some feline cases with increased mast cells had cells detected as basophils, suggesting that mast cells are not lysed in the basophil reagent. The Technicon H1®, Advia 2120®, and Sysmex XT 2000 iV®

have indicated the presence of basophilia when basophilia were not detected by blood smear inspection.³³ Thus an increase in automated basophil counts in the dog and cat should be considered an error. Accuracy of automated basophil counts in other species has not been validated (Dr. Harold Tvedten, personal communication). Alternatively, 100-cell differential counts are imprecise for evaluating basophils, because basophils usually account for less than 2% of the leukocytes in most species. One must count thousands of cells, or use direct counting methods with a hemocytometer and basophil stain to obtain precise counts.

Basophilia

Basophilia in mammals is relatively rare. In healthy animals, basophils are so infrequently seen that they are rarely included in the 100-cell differential cell count. For domestic mammals, only marked or persistent increases in basophil concentrations should be considered abnormal. Typically, when seen, basophilia in domestic mammals is typically associated with an IgE-mediated disorder, and it is usually accompanied by an eosinophilia. Therefore, primary considerations should be given to allergic diseases and parasitism. Causes of basophilia include drugs, foods, inhalants, and parasite stings or bites. Parasites documented to cause basophil recruitment and the accumulation of tissue basophils include ectoparasites such as fleas and ticks, gastrointestinal parasites such as nematodes, and vascular parasites such as *Dirofilaria immitis* and *Dipetalonema reconditum*.¹⁰

If parasites and allergy are ruled out, other inflammatory or neoplastic conditions should be considered. Basophilic leukemia is rare but has been reported in dogs and cats (see Chapter 63). Basophilias may also occur with mast cell neoplasia with or without mastocytosis and in dogs that have lymphomatoid granulomatosis.¹¹ Potentially, basophilia also may occur with other forms of cancer.

Frequently, hyperlipidemia has been mentioned as a cause of basophilia, but there is no good evidence to support this. Basophilia without eosinophilia may indicate that endogenous or exogenous corticosteroids have depressed eosinophil concentrations more than basophil concentrations.

Basopenia

With the reference interval including 0 cells/ μ L, basophils are rarely observed in the blood and bone marrow of healthy animals. Therefore, in most species, basopenia is not recognized. Nevertheless, using more sensitive enumeration techniques, decreased numbers of circulating basophils have been reported in several disorders. Disorders associated with decreased circulating basophil concentrations include urticaria and anaphylaxis, some inflammatory and immunologic conditions, corticosteroid administration, neoplasia, and hemorrhage.

LABORATORY EVALUATION OF MAST CELLS

Structure, Morphology, and Staining Characteristics

When stained with most Romanowsky-type stains, mast cells are identified by their characteristic fine-to-coarse deep purple cytoplasmic granules. However, approximately 15% of the time mast cell granules may stain poorly or not at all when manual quick stains are used. The lack of staining may be associated with dissolution of mast cell granules by these largely aqueous stains if fixation is inadequate. Therefore, leaving the slide in the fixative for at least 5 minutes before staining the slide may improve granule staining.

With Romanowsky-type stains, mast cells are round cells with round to slightly oval, usually central or slightly off-center nuclei. They have variable quantities of cytoplasm that contain varying shades of red to deep purple, variably sized, yet often very coarse granules. If the cytoplasm is not packed with granules, it can appear clear, pale pink, or blue. Nuclei are frequently obscured by the granules, but when seen the chromatin patterns range from coarsely granular to finely stippled, depending on cell maturity. Mast cell nuclei frequently absorb less stain than the granules and thus appear as pale blue circles amongst the abundant mass of deep purple granules. Prominent nucleoli are not typically seen in normal mast cells but may be seen in mast cell neoplasia. In some species, highly granulated basophils can be confused with mast cells.

Mastocythemia and Marrow Mastocytosis

Because mast cells are normal residents of many tissues, occasional mast cells are seen in many tissues including bone marrow and lymph nodes.⁶ Mastocytosis or mastocythemia (also called mastocytemia) may represent either a reactive or a neoplastic state (see Chapter 68 for mast cell neoplasia).

Finding mast cells in the blood of dogs, for many years, was considered highly suggestive of mast cell neoplasia; however, mastocythemia may be seen in animals with inflammatory diseases, regenerative anemia, tissue injury, neoplasia other than mast cell tumors, and necrosis.³⁰ In a study of 19 dogs with mastocythemia, 18 of 19 had acute inflammatory leukograms with associated toxic neutrophils. Mastocythemia also has been associated with inflammatory skin disease in dogs.

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Monocytes and Macrophages and Their Disorders

DOUGLAS J. WEISS and CLEVERSON D. SOUZA

Monocytopoiesis (see Chapter 8)

Morphology (see Section IX)

Structure and Biochemistry

Ultrastructure

Metabolism

Secretion

Cell Surface Receptors

Fc receptors

Complement receptors

Integrin receptors

Toll-like receptors

Mannose receptors

Scavenger receptors

Function

Macrophage Activation

Antimicrobial Mechanisms

Antigen Processing

Regulation of Iron in Phagosomes

Removal of Dead Cells and Damaged Tissue

Interaction with Tumor Cells

Distribution and Fate

Monocyte Disorders

Monocytopenia

Monocyte Dysfunction

Viral infection

Anti-inflammatory response

Monocytosis

Acute monocytic leukemia, acute

myelomonocytic leukemia, chronic

myelomonocytic leukemia (see Chapters 65 and 67)

Macrophage Disorders

Hemophagocytic Syndrome

Chronic Infectious Disease

Idiopathic Systemic Granulomatous Inflammation in Horses and Cattle

Lysosomal Storage Disorders

Inherited

Acquired

Malignant Histiocytosis (see Chapter 73)

Cutaneous/systemic histiocytosis (see Chapter 73)

Localized Histiocytic Sarcoma (see Chapter 73)

Disseminated Histiocytic Sarcoma (i.e. Malignant Histiocytosis, see Chapter 73)

Acronyms and Abbreviations

DNA, deoxyribonucleic acid; FIV, feline immunodeficiency virus; IL, interleukin; MAP, *Mycobacterium avium* subsp. *paratuberculosis*; MHC, major histocompatibility antigen; NF- κ B, nuclear factor-kappaB; Nramp1, natural resistance-associated macrophage protein-1; TGF- β , transforming growth factor-beta; TNF- α , tumor necrosis factor-alpha, TLR, Toll-like receptor; VLA, very late activation antigen.

Monocytes and free and fixed tissue macrophages and their progenitor cells in bone marrow make up the mononuclear phagocyte system.¹³ Dendritic cells are a distinct subset of bone marrow-derived cells that are morphologically and functionally related to macrophages.³ That being said, dendritic cells have been shown to differentiate from monocytes and macrophages in vitro under appropriate cytokine stimulation.³

Monocytes continuously emigrate from the blood into peripheral tissues.^{13,19,36} Depending on the tissue or the inflammatory process encountered, monocytes differentiate into a variety of subtypes of fixed tissue and free, and inflammatory macrophages. Tissue macrophages include Kupffer cells, alveolar macrophages, microglial cells, and osteoclasts (Table 45.1).³⁴ Macrophages in various tissues have specialized functions. For example, alveolar, peritoneal, and pleural

TABLE 45.1 Phenotypic Heterogeneity of Mononuclear Phagocytes Associated With Site-Specific Differentiation In Vivo

Cell Type	Phenotype
Peripheral monocytes	F4/80 ^{low} , CD11b ⁺ , CD68 ⁺ , Dectin-1 ⁺ , CD14 ⁺
Inflammatory monocyte	CCR2 ⁺ , CD62L ⁺ , Ly6C ⁺ , CD14 ⁺
Bone marrow stromal M0/Kupffer cells	F4/80 ⁺ , Sialoadhesin ⁺ , CD68 ⁺ , CD11b ^{low/-}
Splenic red pulp M0	F4/80 ⁺ , CD68 ⁺ , CD11b ^{low/-} , Sialoadhesin ^{low} , MR ⁺ , Dectin-2 ⁺
Splenic white pulp M0	CD68 ⁺
Dendritic cells	CD11c ⁺ , MHCII ⁺ , CD68 ^{low}
Langerhans cells	F4/80 ⁻ , CD11b ⁺ , CD68 ⁺ , Langerin ⁺ , MCHII ⁺ , Dectin-2 ⁺
Inflammatory elicited M0	F4/80 ⁺ , CD11b ⁺ , CD68 ⁺ , MR ⁺ , Dectin-1 ⁺
Resident peritoneal M0	F4/80 ^{high} , CD11b ^{high} , CD68 ⁺ , Dectin-1 ⁺ , DIGNR1 ⁺
Microglia	F4/80 ⁺ , CD11b ⁺ , CD68 ⁺
Alveolar M0	F4/80 ^{low} , CD68 ⁺ , Sialoadhesin ⁺ , MR ⁺ , Dectin-1 ⁺

macrophages have enhanced bactericidal activity, whereas splenic macrophages and Kupffer cells are specialized for phagocytosis of senescent erythrocytes and for iron storage.³⁴ Inflammatory macrophages include epithelioid cells and multinucleate giant cells. Pulmonary intravascular macrophages are a unique part of the mononuclear phagocyte system of several species, including ruminants, pigs, horses, cats, mice, and rats.²¹ These cells localize circulating foreign particles in the lungs.

STRUCTURE AND BIOCHEMISTRY

Ultrastructure

As monocytes develop into macrophages, the cells enlarge, the number of mitochondria and lysosomal granules increase, the size of the Golgi complex increases, and the acid hydrolases content of lysosomes increase (Fig. 45.1). Macrophages are highly dynamic cells usually containing large numbers of cell surface microvilli. These cells are constantly engaged in uptake of fluid through pinocytosis and have variable numbers of small clear vacuoles in their cytoplasm. Phagolysosomes may also be present that contain ingested material. The number and size of mitochondria vary with the phagocytic activity of the cell.

Metabolism

Mononuclear phagocytes derive their basal metabolic energy primarily from glycolysis. Alternatively, oxidative phosphorylation contributes substantially to the generation of adenosine triphosphate in alveolar macrophages. Phagocytosis is accompanied by a marked increase in oxygen consumption which is part of the antimicrobial response. As with neutrophils, macrophages contain the multicomponent NADPH oxidase system that is responsible for the respiratory burst (see Chapter 41). Additional fluids, that may contain nutrients, are continually endocytosed into macrophages by

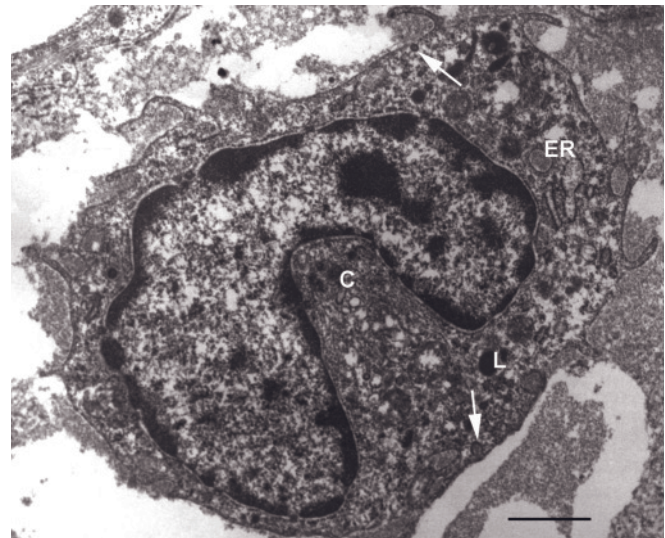


FIGURE 45.1 Porcine tissue macrophage from the skin. Endoplasmic reticulum (ER), primary lysosomes (L), clathrin-coated pits (arrows). Bar, 2 μ m. (Courtesy of W.L. Steffens III, University of Georgia.)

a process of pinocytosis.¹⁵ Fluid uptake occurs through macropinosomes (vesicles larger than 0.2 μ m), clathrin-coated vesicles, and small uncoated vesicles.

Secretion

Macrophages contain a variety of secretory products, most of which are produced after exposure to various stimuli.²⁸ When activated by a variety of agonists, monocytes and macrophages produce proinflammatory cytokines (e.g. interleukin [IL]-1, IL-6, tumor necrosis factor-alpha [TNF- α]), anti-inflammatory cytokines (e.g. IL-10), growth factors (e.g. granulocyte-monocyte colony-stimulating factor, granulocyte colony-stimulating factor, platelet-derived growth factor, and transforming growth factor- β), various enzymes (e.g. collagenase, elastase, acid hydrolases, and

plasminogen activator), alternative and classic complement pathway factors, and prostaglandins and lipoxygenase products.

Cell Surface Receptors

As phagocytes, monocytes and macrophages have a complex array of cell membrane receptors that vary somewhat depending on the tissue of origin and the activation status (Table 45.2).^{13,36} These can be grouped into various functional categories, including adhesion receptors, phagocytosis receptors, growth receptors, differentiation receptors, activation receptors, migration receptors, and function receptors (Table 45.2). However, individual receptors frequently have more than one function. Major receptor groups include Fc receptors, complement receptors, Toll-like receptors (TLRs), major histocompatibility receptors, cytokine receptors, chemokine receptors, and scavenger receptors.³⁶

Fc Receptors

Fc receptors are expressed on the cell surface of monocytes, macrophages, granulocytes, and platelets.³⁶ Three distinct subclasses of Fc receptors have been identified in human cells that interact with various types of monomeric IgG, complexed IgG, or with immune complexes. Human and mouse Fc receptors function to bind the Fc portion of immunoglobulin and function in phagocytosis, cell activation, and in antibody-dependent cellular cytotoxicity. Fc receptors in animal species have been less well investigated but early studies indicate that these receptors may function differently from human Fc receptors. Bovine Fc receptors may function to secrete IgG onto mucosal surfaces. However, Fc receptors do appear to be involved in the activation of bovine neutrophils.^{16,27}

Complement Receptors

Four receptors are present on monocytes and macrophages that bind fragments of C3.⁴⁵ Complement receptor 1 (CD35) binds to C3bi.⁴⁶ The other three complement receptors are members of the integrin family and include CD11a/CD18, CD11b/CD18, and CD11c/CD18. Interaction of complement receptors with complement fragments primarily initiates phagocytosis and to a lesser extent initiates cell activation.

Integrin Receptors

The leukocyte integrin subfamily of adhesion molecules consists of a group of four heterodimeric glycoproteins.⁹ All share a common β_2 -subunit designated CD18 but have variable α -subunits designated CD11a, CD11b, CD11c, and CD11d. CD11a is expressed on all leukocytes; CD11b is present on monocytes, macrophages,

TABLE 45.2 Macrophage Receptors and Their Ligands

Macrophage Receptor	Ligand
Transmembrane receptor	
TLR1:TLR2 heterodimer	Peptidoglycans, lipoproteins, lipoarabinomannan, GPI, zymosan
TLR3	dsRNA
TLR4/CD14/MD2 complex	Lipopolysaccharide (Gram negative bacteria), lipoteichoic acids (Gram positive bacteria)
TLR5	Flagellin from bacteria
TLR7	ssRNA
TLR8	G-rich oligonucleotides
TLR9	Unmethylated CpG DNA from bacteria
TLR11	Propellin
MARCO (macrophage receptor with collagenous structure)	Unopsonized inert particles and bacteria
Scavenger receptors	Unopsonized inert particles and bacteria
Dectin-1	Several types of carbohydrates
DC-SIGN (dendritic cells)	ICAM-2, ICAM-3, lipopolysaccharide
FPR (formyl peptide receptor)	<i>N</i> -formylated peptide of bacteria
MR (mannose receptor)	Mannose, lipopolysaccharide
Cytosolic receptor	
Naip5 (neuronal apoptosis-inhibiting protein 5)	Flagellin
Ipafl	Flagellin?
Nod1 and Nod2 (nucleotide oligomerization domains 1 and 2)	Peptidoglycan
RIG-1 (retinoic acid-inducible gene 1)	dsRNA, dsDNA?
Extracellular receptor	
SP-A and SP-D (surfactant proteins A and D)	ssRNA, lipopolysaccharide, lipoteichoic acids
LPB (lipopolysaccharide-binding protein)	Lipopolysaccharide
CD14	Lipopolysaccharide
MD-2	Lipopolysaccharide
PTX3 (pentraxin 3)	Complement proteins, zymosan
MBL (mannose-binding lectin)	Mannosylated moieties
CRP (C-reactive protein)	Phosphocholine
Complement	Microbial surfaces

and granulocytes; CD11c is primarily present on monocytes, and macrophages; and CD11d is expressed by some macrophages and T cells.²⁶ Several β_1 integrins are also expressed by monocytes and macrophages; these include very late activation antigen (VLA)-4, VLA-5, and VLA-6. These are fibronectin receptors. Integrins along with L-selectins play a central role in cell adhe-

sion to endothelium and extracellular matrix proteins and migration through tissues (see Chapter 41).

Toll-Like Receptors

Mammalian Toll-like (TLR) receptors are a family of proteins that are localized on the cell phagosomal membrane of macrophages and dendritic cells.¹ The major function of these molecules is to detect and bind pathogen-associated molecular patterns and to initiate signal transduction leading to activation of macrophages and dendritic cells. Each of the 11 TLRs detects a different set of microbial components. For example, TLR2 recognizes triacylpeptides; TLR4 in combination with CD14 recognizes lipopolysaccharide, and TLR9 recognizes bacterial DNA.¹

Mannose Receptors

The mannose receptor interacts with linear or branched chain mannosyl and fucosyl residues.³⁶ This permits the mannose receptor to bind a wide variety of bacteria, yeast, and parasitic organisms. The primary result of binding to mannose receptors is phagocytosis; however, some cell signaling via these receptors has been documented.

Scavenger Receptors

Scavenger receptors recognize negatively charged macromolecules.³¹ They have not been extensively studied in animal species. In humans, they have been shown to play a role in uptake of cholesterol from low-density lipoproteins, to recognize pathogens, and to remove apoptotic cells.³¹ Scavenger receptors have been identified in cattle. These scavenger receptors, designated WC1, are transmembrane glycoproteins expressed on gamma delta T cells.⁴ The subpopulation of gamma delta T cells expressing WC1 appear to be inflammatory-type cells, whereas WC1-negative gamma delta T cells appear to be regulatory-type cells.⁴

FUNCTION

A major function of macrophages is to restrict replication of intracellular microorganisms.³⁶ As such, these cells play a key role in defense against a variety of organisms including *Mycobacterium*, *Leishmania*, *Rhodococcus*, *Toxoplasma*, *Listeria*, *Brucella*, *Salmonella*, *Yersinia*, *Shigella*, *Rickettsia*, *Legionella*, and *Theileria*. Other macrophage functional activities include processing and regulating immune responses through presentation of antigens to the immune system and secretion of cytokines (e.g. IL-10, IL-12, transforming growth factor[TGF]- β), modulation of inflammatory responses through secretion of hematopoietic growth factors (e.g. granulocyte-monocyte colony-stimulating factor, granulocyte colony-stimulating factor), initiation of inflammation (e.g. IL-1, IL6, TNF- α), and inhibition of inflammation (e.g. IL-10, TGF- β), production of

cytokines, and chemokines (e.g. CXCL8, CXCL10, CCL5), regulation of iron metabolism, removal of dead and damaged tissue, and interaction with tumor cells (Table 45.3).

Macrophage Activation

When macrophages are activated, they mount specific functional responses that depend on the activating stimuli.²³ When activated by classical inflammatory stimuli like interferon-gamma and lipopolysaccharide, macrophages are proinflammatory and have been termed M1 macrophages (Fig. 45.2). M1 macrophages secrete proinflammatory cytokines, have increased major histocompatibility antigens and costimulatory molecules on their surface, have enhanced endocytic functions, and enhanced capacity to kill organisms. M2 macrophages are alternatively activated macrophages and have anti-inflammatory activity. They have been divided into M2a (activated by IL-4 or IL-13), M2b (activated by immune complexes in combination with IL-1 β or lipopolysaccharide), and M2c (activated by IL-10, TGF- β , or glucocorticoids). M1 macrophages support development of a Th1-type immune response through secretion of IL-12, while M2 macrophages support development of a Th2-type immune response through secretion of IL-10 (see Chapter 51). M2 macrophages are thought to be important in the resolution of inflammation and in tissue repair.²³

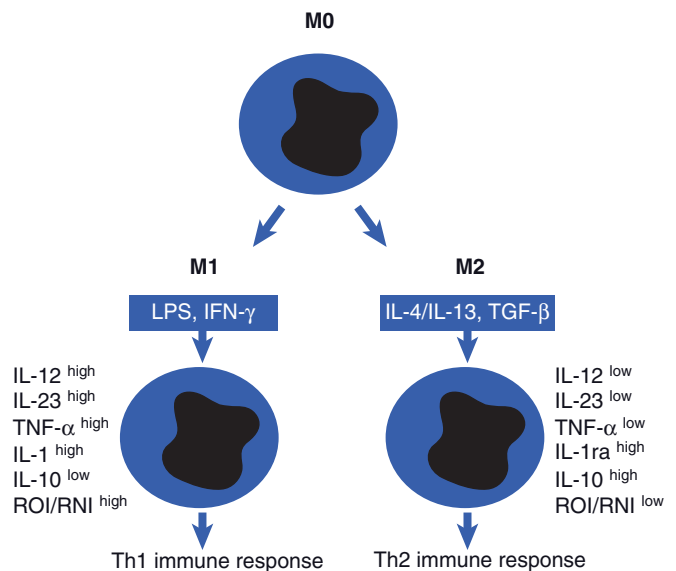


FIGURE 45.2 Macrophage functional activity is dependent on the activating stimulus. Macrophages activated by classical inflammatory stimuli (M1 macrophages) secrete proinflammatory cytokines, induce a Th1-type immune response, and have enhanced antimicrobial activity. Macrophages stimulated by anti-inflammatory stimuli (M2 macrophage) have anti-inflammatory activity, stimulate a Th2-type immune response, and have reduced antimicrobial response. IL, interleukin; RNI, reactive nitrogen intermediates; ROI, reactive oxygen intermediates; TNF- α , tumor necrosis factor- α .

TABLE 45.3 Major Cytokines Expressed by Macrophages

Cytokine	Receptor(s)	Target cell(s)	Major biological function(s)
TNF- α	TNF-R1/R2	Multiple cell and tissues	Proinflammatory; induces apoptosis, cellular proliferation; inhibitory effects on tumorigenesis and viral replication
IL-1	IL 1RI and IL1RI-AcP	Macrophages, T lymphocytes, B lymphocytes, endothelial cells, etc.	Proinflammatory cytokine; promotes costimulation and secretion of cytokines and other acute-phase proteins
IL-6	IL6R α	B lymphocytes, T lymphocytes, thymocytes, myeloid cells, etc.	Proinflammatory and costimulatory action; induces proliferation and differentiation; synergizes with TGF- β to drive Th17 responses
IL-10	IL10R1 and IL10R2	Macrophages, Th1/2 cells, dendritic cells	Immune suppression; down-regulation of MHC class II expression; down-regulates inflammatory responses regulated by Th1 and Th17 cells
IL-12	IL12R β 1 and IL12R β 2	T lymphocytes, NK cells, macrophages	Differentiation and proliferation of T cells; promotes Th1 responses and macrophage activation
IL-18	IL18R and IL18-R-AcP	Th1 cells, NK cells, B lymphocytes	Differentiation and proliferation of Th1 cells; induction of IFN- γ production, enhances NK cell toxicity
IL-19	Same as IL-20	Macrophages, Th1/2 cells, dendritic cells	Biological functions under investigation
IL-20	IL-20R1 and IL-20R2		Regulates proliferation and differentiation of keratinocytes during inflammation; promotes cell expansion of multipotential hematopoietic progenitor cells
IL-23	IL23R and IL23R β 1	T lymphocytes	Inflammatory; promotes proliferation of Th1 and Th17 cells; macrophage activation
IL-27	WSX-1/gp130	T lymphocytes	Induction of early Th1 differentiation by stimulating expression of T-BET transcription factor; inhibition of effector Th17 cell response by inducing STAT-1-dependent blockade of IL-17 production
IL-28	IL28R1/IL10R2	Virus infected cells	Role in immune defense against viruses
IL-33	ST2/IL1R-AcP	T-lymphocytes, mast cells, others?	Costimulation; promotes Th2 cytokine production

Antimicrobial Mechanisms

Our understanding of macrophage antimicrobial mechanisms have developed from study of the interaction of macrophages with several intracellular organisms, the most prominent of which are *Mycobacterium*, *Leishmania*, and *Toxoplasma*.³⁶ These studies have unraveled both the mechanisms by which macrophages kill organisms and mechanisms by which pathogens circumvent these antimicrobial mechanisms. Within veterinary species, most research has focused on the interaction of *Mycobacterium avium* subsp. *paratuberculosis* (MAP, the cause of Johne's disease) with ruminant monocytes and macrophages. Initial studies established that bovine monocyte-derived macrophages lacked the capacity to kill MAP organisms.⁴² Results of more recent studies indicate that cellular responses are initiated when MAP interacts with host cell-membrane receptors.⁴⁴ A major signaling receptor incriminated in susceptibility to MAP infection is TLR2. TLR2 has been shown to activate two key cell signaling pathways, the mitogen-activate protein kinase-p38 pathway and the NF- κ B pathway.⁴⁴ Both pathways initiate transcription of IL-10. Early production of IL-10 appears to suppress macrophage activation and inhibit initiation of a Th1-type

immune response. In addition to failing to initiate inflammatory and immune responses, phagosomes containing MAP fail to acidify and fuse with lysosomes.³⁵ Preincubation of bovine monocyte-derived macrophages with anti-IL-10, anti-TLR2, or an inhibitor of the mitogen-activate protein kinase-p38 pathway resulted in increased phagosome acidification, phagolysosome fusion, and killing of MAP organisms.⁴⁴

Antigen Processing

Once phagocytized, macrophages and dendritic cells degrade complex proteins, attach them to major histocompatibility antigen (MHC) class-II molecules, and transport them to the cell surface for presentation to the immune system. Many virulent organisms interfere with this process, thus attenuating the immune response. Virulent mycobacteria interfere with antigen presentation by inhibiting phagolysosome fusion, preventing activation of proteolytic enzymes in the phagosome, and altering expression and distribution of MHC class-I, MHC class-II, and co-regulatory molecules.²⁹

Regulation of Iron in Phagosomes

The overall role of macrophages in iron metabolism and recycling of red blood cell (RBC) iron is discussed in Chapter 20, and sequestration of iron in the macrophages during inflammatory disease states is discussed in Chapter 37. Here, the role of macrophages in regulating iron within phagosomes will be discussed. Natural resistance-associated macrophage protein-1 (Nramp1, also called SLC11A1) was originally identified in mice and was shown to control resistance to *M. bovis*, *Salmonella typhimurium*, and *Leishmania donovani*.³⁸ Later studies have shown that Nramp1 codes for a 12 transmembrane domain integral membrane phosphoglycoprotein that is expressed by macrophages and neutrophils. Nramp-1 is constitutively expressed, is interferon- γ -inducible, and is rapidly recruited to the phagosome membrane through fusion with Nramp1-containing vesicles.⁷ Nramp1 appears to function by pumping protons and divalent metals out of the phagosome, thereby enhancing phagosome acidification and depleting iron and other divalent metals needed for bacterial growth. Mycobacteria prevent phagosome acidification in part by inhibiting delivery of Nramp1 to the phagosome.

Removal of Dead Cells and Damaged Tissue

Macrophages play a central role in removal of senescent RBCs (see Chapter 22), and in resolution of damaged tissue. Macrophages are essential to wound healing and are attracted to the site of injury by chemotactic signals generated by damaged cells. Lysophosphatidylcholine and other substances released by apoptotic and necrotic cells serve as chemotactic signals for macrophages. At sites of injury, macrophages phagocytize cellular debris and apoptotic cells, recruit inflammatory cells by releasing hematopoietic cytokines and chemokines, and recruit myelofibroblasts.¹⁸ Macrophages also regulate neovascularization, and induce fibrosis.¹⁸ Depletion of macrophages in a mouse model of myocardial injury has been shown to impair wound healing.³⁷

Interaction with Tumor Cells

Many tumors are infiltrated with macrophages. The relationship between macrophages and tumor cells is complex.²² M1-type macrophages can inhibit tumor growth through release of cytotoxic substances such as TNF- α , oxygen radicals, or nitric oxide. Alternatively, M2-type macrophages enhance tumor growth by promoting angiogenesis and tissue repair. Whether macrophages oppose or support tumor growth depends on the activation signals they receive.

DISTRIBUTION AND FATE

The circulating half-life of monocytes varies from 70 hours in humans to 18 hours in mice, to 20–23 hours in cattle. Once released into the blood, monocytes are dis-

tributed in a circulating pool, a marginal pool, and in some species, exist as pulmonary intravascular macrophages. In humans, the ratio of the circulating to marginated pool is 1:3.5. Rabbits have a large marginated pool and approximately 60% of mouse monocytes are marginated.¹² Because of this, stimuli such as infectious diseases may cause monocytosis due to redistribution of the marginated pool into the circulating pool. It is not known if a marginated pool of monocytes exists in other domestic animals; however, monocytosis has been observed after glucocorticoid administration to dogs suggesting that a marginated pool exists in this species.

Monocytes appear to leave the circulation in a random manner. In response to inflammation, monocytes accumulate at the lesion site. This is dependent on integrin and selectin adhesion molecule interaction with receptors expressed on endothelium and chemokines released at the site of inflammation.³⁰ Although macrophages at sites of inflammation have the capacity to divide, less than 5% of the resident macrophage population arise de novo. Free macrophages, found at sites of inflammation, have the capacity to migrate to other tissues and to reenter the circulation.

The exact lifespan of macrophages is unknown but it appears that tissue macrophages are long lived. In human bone marrow transplant recipients, autologous macrophage populations are replaced with donor-derived cells over approximately 100 days.¹² In contrast, studies in mice indicate that mouse macrophages may live for more than 1 year.¹² Unlike tissue macrophages, monocytes and macrophages responding to inflammatory stimuli appear to be short lived cells.

MONOCYTE DISORDERS

Monocytopenia

Cats, horses, and ruminants typically have less than 1,000 monocytes/ μ L and dogs typically have less than 1,500 monocytes/ μ L in the blood. Because of these relatively low numbers, little significance has been attached to monocytopenia. Due to the long lifespan of tissue macrophages and their capacity to replicate de novo, even prolonged suppression of monocytopenia is relatively inconsequential. Monocytopenia is observed in aplastic anemia associated with a variety of causes including estrogen toxicity, chemotherapy, and parvovirus infection (see Chapter 39). Because monocytes are produced in 3 days from bone marrow progenitor cells, whereas neutrophils require 6 days to develop, recovery from the monocytopenia occurs before recovery from a neutropenia. Therefore, monitoring the monocyte count may be useful in predicting recovery from bone marrow suppression.

Monocyte Dysfunction

Viral Infection

Several viruses infect monocytes and macrophages, and can alter cell function without altering cell numbers.

Many lentiviruses have a tropism for mononuclear phagocytes. Lesions produced are generally attributable to altered cytokine secretion and defective anti-inflammatory activities. Feline immunodeficiency virus infects monocytes and macrophages as well as CD4+ T cells.²⁰ Peritoneal macrophages from feline immunodeficiency virus-infected cats produced less IL-1, suggesting that they have altered function.

Infection of monocytes and macrophages has also been described with the sheep (Maedi-Visna virus), goat (caprine arthritis encephalitis virus), cattle (bovine immunodeficiency virus), and horse (equine infectious anemia) lentiviruses.^{5,24,33} Although various cell types are susceptible to infection with the sheep lentivirus, only macrophages are permissive for viral replication.⁵ Infection by the goat lentivirus alters expression of cytokines by infected macrophages.¹⁷ Blood monocytes are susceptible to infection with equine infectious anemia virus (see Chapters 19, 32, and 35). Little effect on monocyte function has been observed in experimental infections of cattle with bovine immunodeficiency virus, but this may reflect the low levels of infection achieved.³³

Several other viruses infect macrophages, including many herpesviruses. This infection results in recurrent inflammatory lesions that may result from aberrant inflammatory cytokine production. Infection of cat macrophages with feline leukemia virus subgroup C results in increased production of TNF- α that has been shown to suppress erythropoiesis *in vitro*.¹⁶ Therefore macrophage infection may contribute to the erythroid hypoplasia/aplasia associated with feline leukemia virus infection (see Chapter 38).

Anti-Inflammatory Response

Monocytes and lymphocytes in many humans and dogs with septic and non-septic inflammatory diseases have decreased function, as determined by detecting decreased MHC class II expression, suggesting impaired immune responsiveness.⁴⁵ When stimulated with various agonists, these monocytes tend to produce predominantly anti-inflammatory cytokines. This state is thought to represent a compensatory anti-inflammatory response. The purpose of this anti-inflammatory response is to prevent an uncontrolled systemic inflammatory response that could lead to respiratory distress syndrome and multiple organ dysfunction.

Monocytosis

Monocytosis can result from a reactive (i.e. benign) condition or a malignant condition. Malignant proliferations of monocytic cells, including acute and chronic monocytic leukemia and acute myelomonocytic leukemia, are discussed in Chapters 65 and 67. Reactive monocytosis generally results in a modest increase in monocyte numbers with monocyte counts rarely exceeding 10,000/ μ L. In dogs, the most frequent cause of monocytosis is excess endogenous or exogenous corticosteroids.⁴⁷ Alternatively, monocytopenia occurs in

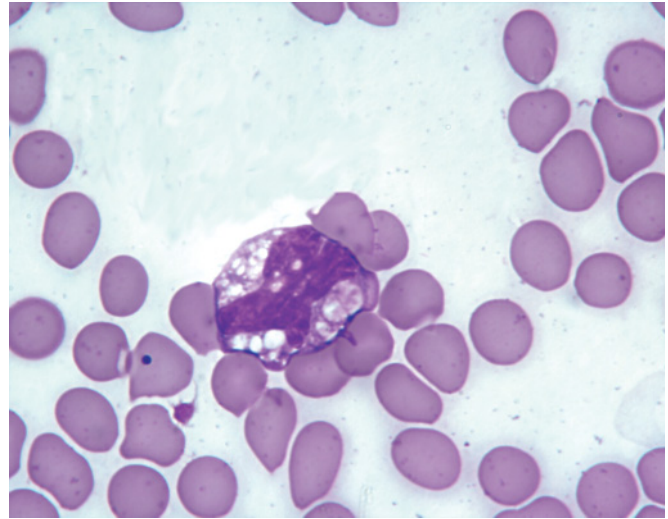


FIGURE 45.3 Reactive monocyte from a dog with bacteremia. Monocytes comprised 22% of the differential WBC count. The cytoplasm of all monocytes was highly vacuolated and the nuclei were convoluted. Wright's stain. (Courtesy of Dorothy Bienzle, University of Guelph.)

humans and laboratory animals in response to corticosteroids; cats, horses, and cow monocytes have a variable response to corticosteroids.¹⁹ Other causes of reactive monocytosis include infections and necrosis. Infectious causes include acute and chronic bacterial infections, rickettsial infections, fungal infections, and protozoal infections. Monocytosis is not seen as consistently as is neutrophilia in infectious diseases. Monocytosis has been documented in dogs with bacterial endocarditis, chronic mycobacteriosis, and ehrlichiosis.^{8,47} Necrotic disorders associated with monocytosis include hemorrhage, hemolysis, malignant neoplasia, infarction, and trauma. In necrotic conditions, monocytes/macrophages are needed to phagocytize necrotic debris and initiate wound healing. Morphologic alterations may be seen in blood monocytes in reactive monocytosis. Typically, reactive monocytes have highly vacuolated cytoplasm (Fig. 45.3)

MACROPHAGE DISORDERS

Classification of macrophage disorders is problematic. Benign macrophage proliferation can result from macrophage activation syndrome (i.e. hemophagocytic syndrome), lipid storage disorders, or occur secondary to several chronic infectious diseases. An idiopathic systemic granulomatous disease has also been described in horses and cattle.^{2,32} Malignant processes are difficult to differentiate from reactive processes and include histiocytoma, and localized and systemic histiocytic sarcoma (see Chapter 73).¹¹ Other histiocytic conditions, including cutaneous/systemic histiocytosis, and hemophagocytic histiocytosis are more difficult to categorize as to whether they are malignant or benign conditions (see Chapter 73).

Hemophagocytic Syndrome

Hemophagocytic syndrome is a benign proliferative disorder of activated macrophages that is associated with multiple cytopenias in the blood.^{39,41} The pathogenesis of hemophagocytic syndrome remains elusive. Cytopenias associated with hemophagocytic syndrome are thought to result from phagocytosis of hemic cells because bone marrow is usually hypercellular. Among human patients, hemophagocytic syndrome is divided into two subcategories: primary hemophagocytic lymphohistiocytosis and macrophage activation syndrome.¹⁴ Primary hemophagocytic lymphohistiocytosis is a group of autosomal recessive immune disorders in which approximately one-third of patients have a mutation in the gene encoding perforin,¹⁴ a protein that mediates the cytotoxic activity of natural killer cells and cytotoxic T cells. This suppresses natural killer activity and results in lack of control of cellular immune responses. Over secretion of Th1-type cytokines may cause activation and proliferation of macrophages. Resultant secretion of proinflammatory cytokines, including TNF- α , IL-1, and IL-6, further amplifies the inflammatory process. Although natural killer and cytotoxic T cells have been incriminated in macrophage activation syndrome as well, the mechanism is poorly understood.¹⁴

Hemophagocytic syndrome has been documented in dogs and cats.⁴¹ In a retrospective study, 3.9% of canine clinical bone marrow specimens, evaluated at a veterinary teaching hospital, had evidence of hemophagocytic syndrome.⁴¹ Alternatively, hemophagocytic syndrome appears to be a relatively rare condition in cats. Clinical signs associated with canine hemophagocytic syndrome include fever, icterus, splenomegaly, hepatomegaly, and diarrhea. In dogs, hemophagocytic syndrome is associated with immune-mediated, infectious, and neoplastic/myelodysplastic conditions, and also occurs as an idiopathic condition. Overall, dogs with infection-associated hemophagocytic syndrome appear to have a better survival rate than dogs with immune-associated and idiopathic hemophagocytic syndrome.

Hemophagocytic syndrome is difficult to diagnose both clinically and cytologically. Because the condition frequently occurs secondary to other diseases and clinical signs resemble those associated with bacterial sepsis and the systemic inflammatory response syndrome, the condition may be underdiagnosed.^{39,41} Further, cytopenias may be attributed to excessive demand for neutrophils in tissues or consumption of platelets due to disseminated intravascular coagulopathy. Therefore, it is important to determine which cases need bone marrow evaluation for detection of hemophagocytic syndrome. In human medicine, serum ferritin concentration has been used as a screening test for detection of hemophagocytic syndrome and malignant histiocytosis. Serum ferritin has been evaluated as a diagnostic test in dogs.¹⁰ Moderate hyperferritinemia was observed in hemolymphatic neoplasia, and hepatic disease. Marked hyperferritinemia was observed in malignant

histiocytosis and in immune-mediated hemolytic disease. Therefore, serum ferritin concentration appears to be very high in several pathologic conditions in dogs.

Pathologic conditions that can be confused with hemophagocytic syndrome include malignant histiocytosis, necrosis, granulomatous inflammation, and monocytic and myelomonocytic leukemias.^{6,40,43} Malignant histiocytosis (also termed disseminated histiocytic sarcoma) is a malignant proliferation of myeloid dendritic cells.⁶ Features of malignant histiocytosis that are useful in differentiating it from hemophagocytic syndrome include highly anaplastic features, large cell size, multinucleate giant cells, and greater than 3% histiocytic cells in cellular bone marrow aspirates. Additional tests to differentiate hemophagocytic macrophages from malignant dendritic cells include evaluation of flow cytometric scatter plots and immunophenotyping.⁴¹

Chronic Infectious Disease

Several chronic systemic infectious diseases result in proliferation of inflammatory macrophages with or without multinucleate giant cells or epithelioid cells in tissues. In some cases activated macrophages and epithelioid cells can be mistaken for a malignant process. Conditions frequently associated with macrophage proliferations include mycobacterial infections, deep fungal infections (e.g. *Blastomyces*, *Histoplasma*), and other systemic fungal infections.

Idiopathic Systemic Granulomatous Inflammation in Horses and Cattle

Idiopathic systemic granulomatous inflammation is characterized by perivascular infiltrates of macrophages, lymphocytes, plasma cells, and giant cells in multiple organs.^{2,32} Typically nodules are spread throughout the body. Bacterial, viral and fungal causes have been ruled out and an immune-mediated cause is suspected.

Lysosomal Storage Disorders

A variety of inherited lysosomal storage disorders have been described in animals. These disorders are classified into broad groups based on the type of macromolecule that accumulates within lysosomes.²⁵ Although progressive neurological disease is the major clinical disease in most of these disorders, hematologic alterations may be encountered associated with splenomegaly, cytopenias, and the accumulation of swollen foamy macrophages in various tissues. Hereditary disorders include GM1 and GM2 gangliosidosis, glucocerebrosidosis (Gaucher disease), sphingomyelinosis (Neimann-Pick disease), galactosialidosis, galactocerebrosidosis (globoid cell leukodystrophy, Krabbe disease), α - and β -mannosidosis, and α -L-fucosidosis.

In addition to the inherited lysosomal storage disorders, several acquired lysosomal storage disorders have

been described. Swainsonine is a toxic agent in several species of plants. This indolizidine alkaloid is a potent inhibitor of lysosomal α -mannosidase. Therefore, prolonged ingestion of these plants by ruminants results in a disease process very similar the hereditary form of α -mannosidosis. Similarly, ingestion of *Trachyandra diovaricata*, *Phalaris* sp. or *Solanum* has been associated with lysosomal storage disorders.

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Interpretation of Ruminant Leukocyte Responses

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Changes in Leukocyte Morphology
 Physiologic Effects on the Leukogram
 Age-related Leukogram Changes
 Stress and Excitement-related Leukogram Changes
 Parturition
 Pathologic Effects on the Leukogram
 Inflammation
 Leukocyte Organisms

Neutrophilia and Neutropenia
 Eosinophilia and Eosinopenia
 Basophilia and Basopenia
 Lymphocytosis and Lymphopenia
 Monocytosis and Monocytopenia
 Camelid Leukocyte Responses

Acronyms and Abbreviations

BLAD, bovine leukocyte adhesion deficiency; BLV, bovine leukemia virus; BVDV, bovine viral diarrhea virus; JLIDS, juvenile llama immunodeficiency syndrome; N:L, neutrophil to lymphocyte ratio; WBC, white blood cell.

Interpretation of ruminant white blood cell (WBC) responses, like that of other species, can be an essential component of diagnosis, monitoring, and prognosis of disease, though a single leukogram is rarely diagnostic for a specific condition. In some respects ruminant WBC responses are similar to those in other species, but they also have some distinct features.

Cattle normally have the lowest neutrophil to lymphocyte ratio (N:L) of the domestic species with a ratio of about 0.5 in adult cattle.⁹⁴ Sheep have a similar N:L to cattle. Adult goats 3 years of age and older have an N:L of approximately 1.0, though younger goats may have an N:L like that of cattle and sheep.³⁵ These ratios frequently change in a number of physiologic and pathologic conditions.

Ruminants mobilize bone marrow stem cell reserves more slowly than, for example, dogs though production time for neutrophils is approximately 1 week in both calves and dogs.¹⁰¹ This contributes to the unique ruminant early leukogram response to acute inflammation. Cattle, sheep, and goats also typically have a lower peak level of leukocytosis in the face of acute inflammation than do other domestic animals.¹⁰¹ For cattle, a WBC count of 20,000–30,000/ μL is considered extreme leukocytosis.⁴⁰ Inflammatory conditions in goats can result in a leukocytosis of 22,000–27,000/ μL .⁴⁰

CHANGES IN LEUKOCYTE MORPHOLOGY

Morphologic changes in neutrophils may occur when cells develop in the bone marrow in the face of acute, severe inflammation. These changes, characterized as toxic, include increased cytoplasmic basophilia from retained ribosomes, azurophilic (“toxic”) granules that are retained primary granules, and cytoplasmic vacuolation (Figs. 46.1 and 46.2).⁴¹ Döhle bodies, which are retained rough endoplasmic reticulum, appear as distinct bluish cytoplasmic structures in some toxic neutrophils.

Pseudo Pelger-Huët anomaly is another morphologic change that can occur transiently in ruminants with inflammatory disease. This is an infrequent consequence of impaired granulopoiesis in which granulocyte nuclei do not segment normally. This results in band-shaped to round nuclei that have mature-appearing chromatin.⁷⁰

Large, abnormal-appearing granules appear in neutrophils, eosinophils, and basophils of cattle with Chediak-Higashi syndrome (see Chapter 42). The number of abnormal granules appears variable from one animal to the next on Wright-Giemsa-stained blood smears.⁸ However, staining with Sudan black B or peroxidase shows more uniform granule abnormalities in

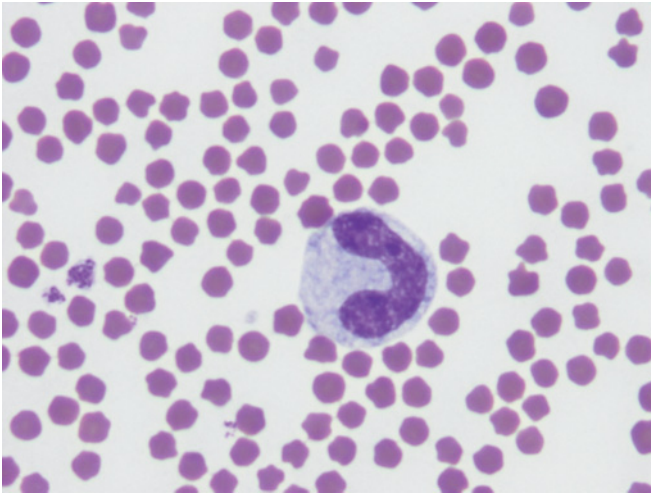


FIGURE 46.1 Toxic band, caprine blood. Wright-Giemsa stain.

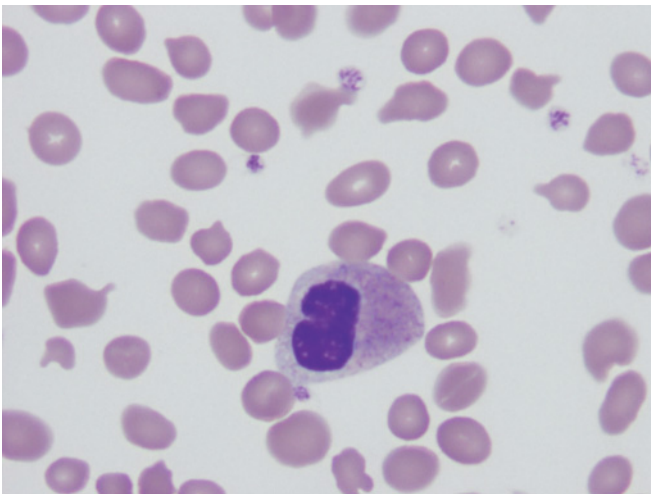


FIGURE 46.2 Toxic metamyelocyte, calf blood. Wright-Giemsa stain.

affected animals.⁶⁸ The abnormal granules are defective lysosomal granules that have decreased function.⁷² This inherited disorder has been reported in Japanese Black, Brangus, and Hereford cattle.

PHYSIOLOGIC EFFECTS ON THE LEUKOGRAM

Age-Related Leukogram Changes

Some studies report that neonatal calves have total leukocyte counts that are higher than adult reference intervals, but others report mean WBC counts in neonates are within adult reference intervals.^{20,50,65,97} The difference may reflect marked variability between individual calves. Calves have a N:L ratio greater than 1.0 at birth, with a rapid decrease in neutrophils and more modest increase in lymphocytes resulting in an N:L ratio similar to that of adults by a week of age.^{40,41,96} This rapid shift

may be related to the stress of birth and release of cortisol, as it is not seen in calves delivered by cesarean section.⁴⁰ A gradual decrease in lymphocyte numbers is then seen as cattle age.^{65,104} Eosinophil percentages are relatively low at birth, show increases by 3 weeks of age and reach adult levels by 2 years of age.^{20,105}

Age-related changes in lambs are reported to be similar to those in calves, with a rapid decline in neutrophil numbers and an increase in lymphocyte numbers reversing the N:L of greater than 1.0 that is present at the time of birth.⁹⁸

Young goats experience an increase in total WBC numbers from birth to a peak at 3 months that reflects both significant increase in lymphocyte numbers and a smaller increase in neutrophil numbers that peak at 1 month of age.³⁵ The N:L ratio which is greater than 1.0 at the time of birth, reaches a nadir of 0.3 in 3 month-old kids, but then increases back to about 1.0 in adults.⁸⁸ Some studies show a gradual increase in eosinophil counts from young kids to adult goats.⁸⁷

Stress and Excitement-Related Leukogram Changes

A stress leukogram is frequently present in ruminants that are exposed to endogenous or exogenous glucocorticoids. Leukogram changes include a mild mature neutrophilia, lymphopenia, eosinopenia, and variable monocytosis.^{3,52} In cattle, the N:L ratio is frequently reversed to greater than 1.0. Several studies have shown that certain subpopulations of circulating lymphocytes are disproportionately reduced in experimentally induced stress. The percentage of $\gamma\delta$ T cells have been shown to decrease in cattle given immunosuppressive doses of dexamethasone with more variable effects on B cells and other T cell populations.^{3,64} Decreased proliferative capability of lymphocytes is observed in cattle treated with dexamethasone.^{3,64} The mechanisms for corticosteroid-induced neutrophilia are postulated to include increased release of neutrophils from the marginated pool, accelerated movement of neutrophils from the bone marrow to the blood, and decreased diapedesis of neutrophils from the blood to tissues.⁴⁰ Studies of the effects of dexamethasone on expression of neutrophil adhesion molecules and other proteins have begun to clarify these mechanisms.⁷ For example, shedding of neutrophil surface L-selectin and CD18 adhesion receptors occurs in response to injection of dexamethasone.⁷¹ However, there may be differences in naturally-induced stress and dexamethasone-induced effects on neutrophils.⁵⁵ Transportation stress in goats and cattle has been shown to result in neutrophilia, lymphopenia and a marked increase in the N:L ratio like that seen in dexamethasone-induced stress. The changes have been reported as transient, but persisting for up to 24 hours following transport.^{43,57}

Physiologic leukocytosis is a transient increase in neutrophils and lymphocytes caused by epinephrine released in the face of excitement or fear. The neutrophils are mature and the changes are present for a short period of time on the order of 30 minutes following the stimulus.⁴⁰

Parturition

In the periparturient period, cows typically have a stress leukogram characterized by a neutrophilia, lymphopenia, eosinopenia, and monocytosis. Several studies have shown shifts in proportions of different lymphocyte populations at the time of parturition. These changes may be partly related to nutritional status and other health parameters, but pregnancy and lactation appear to have the primary effect.^{63,102} Decreased *in vitro* function of neutrophils and lymphocytes from periparturient cows contribute to an immune-suppressed state that predisposes cows to infectious diseases at this time (see Chapter 42).⁴⁶ These functional deficiencies reach a nadir during the first week post-partum.

Sheep and goats also experience leukocytosis associated with marked mature neutrophilia, lymphopenia, and mild eosinopenia at the time of parturition.^{4,62} In sheep, these changes resolve to pre-partum levels by the 14th day post-partum.⁴

PATHOLOGIC EFFECTS ON THE LEUKOGRAM

Inflammation

The early leukogram changes in acute inflammation are probably the most distinctive feature of ruminant WBC responses. In the first 24–48 hours of acute, severe inflammation, a marked neutropenia occurs due to recruitment of neutrophils from the blood to the site of inflammation and slower mobilization of the marrow stem cell pool compared to other species.^{58,101} The effect of stress-induced corticosteroids may also contribute to an overall leukopenia with a decrease in lymphocyte numbers. The relatively larger decrease in neutrophil numbers compared to lymphocytes, results in a lowered N:L ratio. At about 24 hours following the onset of the inflammatory insult, immature neutrophils including bands, metamyelocytes, and sometimes myelocytes begin to appear in the blood and may show signs of toxicity (Fig. 46.3). This left shift in the face of neutropenia, suggests a poor prognosis in most species. However, ruminants can rebound with an increase in neutrophil numbers in about 3–5 days. This increase includes both immature and mature neutrophils at this time, and eventually, bone marrow production of mature neutrophils is increased enough to result in a mature neutrophilia in the peripheral blood. Neutropenia with a left shift that persists beyond several days indicates failure of the bone marrow to respond normally.⁴⁰

Inflammation that becomes chronic may be associated with neutrophilia or normal neutrophil numbers. The lack of a predictable neutrophilia in chronic inflammation and in acute inflammation that is less severe, makes plasma hyperfibrinogenemia a better indicator of these conditions in ruminants. Cattle and sheep may have increases in plasma fibrinogen of up to 1.5 g/dL or higher, while the response is less in goats.⁴⁰ Monocytosis may be seen in either acute or chronic

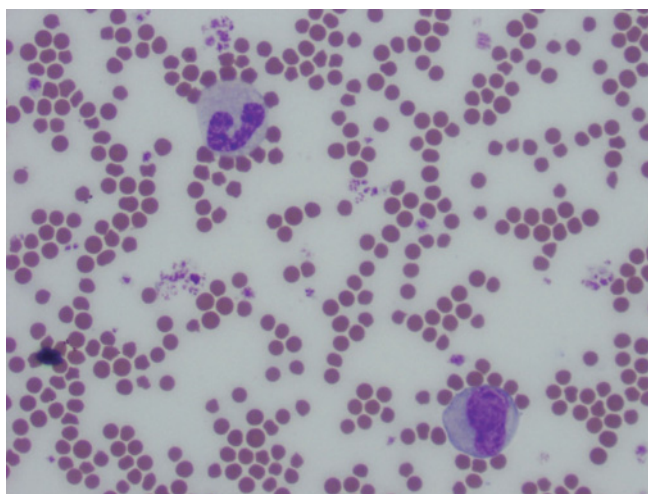


FIGURE 46.3 Left shift, caprine blood, band and metamyelocyte. Wright-Giemsa stain.

inflammation in ruminants, but it is not a consistent change.

Leukocyte Organisms

Anaplasma phagocytophilum is an obligate intracellular bacterium formerly known as *Ehrlichia phagocytophila*.^{106,108} It infects neutrophils, eosinophils, and monocytes of cattle, sheep, and goats as well as other mammals.⁹² The organism appears as small dark-blue to purple cocci in the cytoplasm of infected WBCs in blood smears. Microcolonies form morulae that may also be seen in the cytoplasm of WBCs.⁷⁶ Organisms are carried by ticks. Clinical signs of infection may include transient fever, lameness, abortion, and reduced milk yield in dairy cattle. Infection may also cause immune suppression by avoiding killing by neutrophils and decreasing neutrophil and lymphocyte function; thus secondary infections may be seen. At peak bacteremia, more than 50% of the host's granulocytes may be infected.¹⁰⁷ Sheep infected with *A. phagocytophilum* are frequently severely leukopenic, due to both neutropenia and prolonged lymphopenia. Lymphocyte subsets are differentially affected, with early reductions in CD4+ T cells and $\gamma\delta$ T cells and a relative increase in CD8+ T cells.^{104,105} The organism has multiple effects on neutrophil function, including inhibition of phagosome-lysosome fusion, suppression of the respiratory burst and delay of neutrophil apoptosis, which effectively allows the organism to finish replication in a cell which normally has a short lifespan.¹⁰⁷

NEUTROPHILIA AND NEUTROPENIA

Neutrophilia in ruminants is frequent in mild or moderate inflammation and following the acute stage of more severe inflammation as the bone marrow increases neutrophil production. In chronic inflammation, either a neutrophilia or normal neutrophil count may be

observed.^{19,74} Neutrophilia associated with inflammation may or may not include a left shift, toxic changes, and hyperfibrinogenemia. Inflammatory neutrophilia has been reported in bacterial, viral, fungal, protozoal, and parasitic infections of cattle, goats, and sheep.^{1,6,60,82,84,93,96} Sites of infection in these reports include mammary gland, liver, central nervous system, heart, gastrointestinal tract, respiratory tract and urinary tract.^{5,19,30,54,74,96}

Neutrophilia is also variably reported in cases of neoplasia,⁴⁹ abomasal displacement,⁷⁹ toxicosis,¹⁵ immune-mediated anemia,⁵¹ acute respiratory distress syndrome associated with hypersensitivity,⁹⁹ and in cattle treated with somatotropin.²²

Bovine leukocyte adhesion deficiency (BLAD) is an inherited disorder of cattle that results from defective expression of $\beta 2$ integrins on leukocytes (see Chapter 42).⁶⁷ The defective adhesion molecules result in a primary immunodeficiency characterized by decreased neutrophil function. Affected cattle have multiple recurrent or chronic infections as well as delayed wound healing. The condition is associated with a persistent moderate to marked neutrophilia with no evidence of morphologic abnormalities in neutrophils.^{47,67} A left shift or toxic changes in neutrophils might be seen due to secondary infections.

Physiologic causes of neutrophilia are frequent: the effects of stress and excitement were described in a previous section.

Neutropenia is frequently seen in ruminants with peracute and acute severe inflammatory diseases including Gram-negative sepsis,⁸¹ mastitis, peritonitis, metritis, pneumonia, and gastrointestinal disease. The neutropenia usually begins to resolve after 48 hours as bone marrow releases immature and eventually mature neutrophils. Neutropenia that continues beyond 3–4 days indicates that granulopoiesis is suppressed or is inadequate to meet the demand for neutrophils.⁴⁰

Several viral infections of ruminants cause neutropenia. These include bovine viral diarrhea virus (BVDV), border disease virus, and bluetongue virus.^{36,48,59,89} The neutropenia is transient in most viral infections. The mechanism of neutropenia in high virulence and low virulence isolates of a noncytopathic type 2 BVDV was studied. While both induced neutropenia, the high virulence isolate caused a more prolonged neutropenia with evidence of delayed myelopoiesis. In vitro studies showed that the high virulence isolate significantly decreased the proliferative capacity of bone marrow progenitor cells.⁴⁸ Additional mechanisms of neutropenia may include direct infection of progenitor cells as well as loss of neutrophils into inflamed organs.⁸⁹

Organisms in the Anaplasmataceae family include *Anaplasma phagocytophilum* and *Ehrlichia* (formerly *Cowdria*) *ruminantium*, the agent of heartwater disease of ruminants. Neutropenia is a consistent feature of acute infection with these agents, as it is with other ehrlichiae.^{38,56,76} Although neutrophils are one of the target cells for *A. phagocytophilum*, this is not the case for *Ehrlichia ruminantium*, which infects endothelial cells.

Neutropenia also has been reported in cases of theileriosis,⁶⁹ experimental mycoplasmosis,⁷⁷ *Psoroptes ovis* infestation,⁹¹ and trypanosomiasis.¹ In experimentally-induced trypanosomiasis in calves, neutropenia was severe for 14 weeks following infection. This was associated with a reduction in myeloid precursors in bone marrow.¹⁰⁰

Bone marrow necrosis, fibrosis, or suppression will lead to neutropenia in association with other cytopenias. These have been reported in association with toxins such as bracken fern,⁷³ dioxin,²⁵ furazolidine,⁸³ hydroxyurea,⁸⁶ and the tropical plant *Ipomoea carnea*⁹⁵ and may also be idiopathic.^{2,104}

EOSINOPHILIA AND EOSINOPENIA

Eosinophils are crucial cells in host response to parasitic infections and in allergic reactions. Eosinophilia has been reported in a variety of endoparasitic infections in goats,^{30,33,53,80,109} cattle,¹⁴ and sheep.⁸⁰ Eosinophilia is not always present in parasitic infections even when there is an eosinophil response in tissues.⁴⁴ Ectoparasites can elicit an eosinophilia as well,³⁹ though one study showed that cattle experimentally infested with ticks did not have increased peripheral eosinophil numbers when compared to controls.¹⁰

Eosinopenia may be a component of a stress response in ruminants. Extreme eosinopenia has also been reported in *Theileria parva* and *Theileria annulata* infections in cattle.^{62,69} Toxins and other causes of bone marrow necrosis, fibrosis, or suppression may result in pancytopenia that includes eosinopenia.

BASOPHILIA AND BASOPENIA

In general, basophils react similarly to eosinophils, that is, they tend to increase in response to parasitic infections and hypersensitivity; however, significant differences in the basophil count are not frequently reported in ruminants. Basophilia has been reported in cattle with tick infestations^{10,107} and in goats experimentally infected with nematodes.⁷⁵ Cattle infected with flukes showed no increase in basophil numbers¹⁴ and sheep appear to be less likely to display basophilia than some other species.⁷⁸ Basophil numbers are normally so low in the circulation that basopenia is infrequently reported.

LYMPHOCYTOSIS AND LYMPHOPENIA

In general, lymphocytosis is not common in ruminants, though it may occur in chronic viral infections, chronic trypanosomiasis, and in other causes of chronic inflammation as well as epinephrine release and lymphocytic leukemia.

The bovine leukemia virus (BLV) causes a persistent lymphocytosis in approximately one-third of infected cattle (see Chapter 62).^{29,42} Approximately 3–4% of infected cattle eventually develop leukemia. In contrast, sheep experimentally infected with BLV do not develop

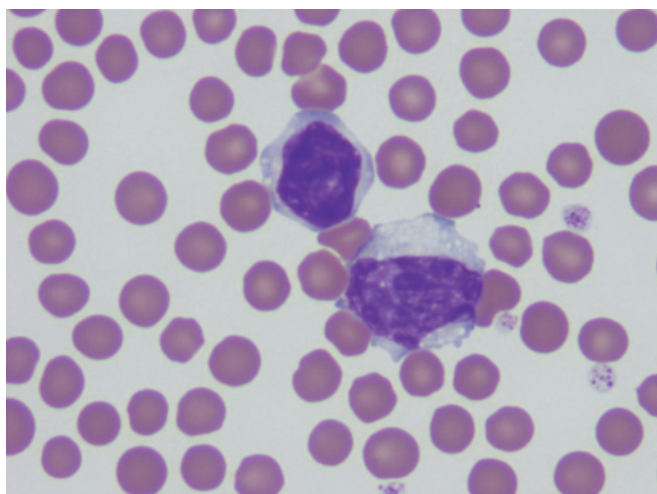


FIGURE 46.4 Circulating lymphoblast, bovine leukemia virus-positive cow. Wright-Giemsa stain.

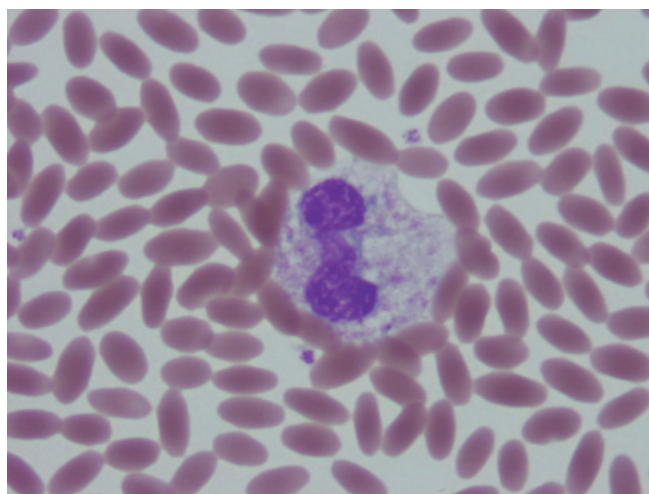


FIGURE 46.5 Toxic band, llama blood. Wright-Giemsa stain.

persistent lymphocytosis and nearly 100% develop leukemia (Fig. 46.4).²⁴ Other species have been infected with the virus, but none have been shown to develop a persistent lymphocytosis or leukemia. The lymphocytosis in BLV-infected cattle is characterized by the accumulation of a polyclonal population of B cells which predominantly express IgM and are CD5+ and CD11b+.^{17,23,61} In cattle, lymphocytosis results from decreased B cell apoptosis.¹⁷ In contrast to BLV infected cattle, leukemia in sheep develops from increased monoclonal B cell proliferation.^{16,18}

The most frequent cause of lymphopenia in ruminants is the corticosteroid-induced stress response. Lymphopenia may also occur in the acute phase of viral, ehrlichial, mycoplasmal or other bacterial infection and septicemia.^{11,45}

MONOCYTOSIS AND MONOCYTOPENIA

Monocytosis is sometimes seen as part of a stress response in ruminants, but not as frequently as in other species. It may also be noted in several inflammatory conditions.¹⁰⁵ Monocytopenia may be associated with endotoxemia and other peracute and acute inflammation.⁴⁰

CAMELID LEUKOCYTE RESPONSES

The Camelidae, which include llamas, alpacas, guanacos, vicunas, and the Old World camels, are sometimes considered to be modified ruminants. Hematologically, they are different from the ruminants in several ways. Reference intervals for total WBC numbers and for eosinophils are higher for healthy adult camelids than they are for cattle, sheep, and goats.^{26,103} The N:L ratio in camelids is reported to vary, with an average of about 1.5 which is more similar to horses than to cattle and sheep.^{21,26,27,32,103} The differences in the reported ratios

have been attributed to epinephrine release causing a transient increase in neutrophils at the time of capture and sampling. The typical features of a stress leukogram including mature neutrophilia and lymphopenia are seen in camelids, and epinephrine-induced or physiologic neutrophilia and lymphocytosis are also seen to varying degrees.^{26,103}

Camelids with inflammatory conditions most frequently have a neutrophilia that may or may not be accompanied by a left shift and hyperfibrinogenemia.³⁴ Neutrophilia may be higher than that seen in ruminants. In experimental infection of alpacas with *Corynebacterium pseudotuberculosis*, total WBC counts reached as high as 30,000/ μ L, mainly due to increased numbers of neutrophils.⁹ An initial neutropenia may be seen in acute inflammation, particularly in endotoxemic and septic camelids; this tends to rebound to a left shift and neutrophilia in 12–24 hours (Fig. 46.5).²⁸

A left shift was a consistent leukogram finding in 10 cases of lymphosarcoma in llamas and alpacas.¹² A case of acute myeloid leukemia in an alpaca was characterized by a neutropenia with a left shift and toxic changes in the neutrophils.⁹⁰ Myelodysplasia in an alpaca was accompanied by a severe leukopenia that was primarily due to lymphopenia.⁶⁶

In a series of cases of llamas with juvenile llama immunodeficiency syndrome (JLIDS), affected animals had leukograms that varied from severe, left-shifted neutropenia to mature neutrophilia.^{37,85} It was suggested that the variability reflected the degree of secondary inflammation and disease at the time of JLIDS diagnosis. Interestingly, lymphopenia is not a feature of the syndrome.³⁷

Eosinophil numbers tend to be higher in normal camelids than in other domestic species. This has not been proven to be related to external or internal parasitism. Parasite infections may be associated with eosinophilia in camelids.³⁴ However, eosinopenia was actually a very frequent finding in a series of llamas and alpacas with the intestinal coccidian parasite *Eimeria*

macusaniensis.¹³ In addition to eosinopenia, most of the camelids had neutrophilia, a frequent left shift, and monocytosis. It may be that animals with clinical signs associated with *Eimeria macusaniensis*, are stressed, and thus immune-compromised. Leukogram findings then represent both inflammation and stress. Leukocytosis due to lymphocytosis and monocytosis has been reported in trypanosomiasis in camels, with no accompanying eosinophilia.³¹

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Interpretation of Equine Leukocyte Responses

ELIZABETH G. WELLES

General Considerations in Equine Leukogram Interpretation
 Cell Morphology (see Chapter 106 for normal morphology)
 Age, Gender, and Exercise Related Leukogram Changes
 Leukogram Response Patterns
 Leukograms Characterized by Leukocytosis
 Physiologic leukocytosis
 Stress and corticosteroid effects

Inflammation/infection-associated effects
 Eosinophilia
 Lymphocytosis
 Leukemias (see Section V)
 Leukograms Characterized by Cytopenias
 Lymphopenia
 Monocytopenia, eosinopenia, and basopenia
 Neutropenia
 Benefit of serial leukograms

Acronyms and Abbreviations

CBC, complete blood count; EDTA, ethylenediaminetetraacetic acid; IgG, immunoglobulin G; IgM, immunoglobulin M; IL, interleukin; RNA, ribonucleic acid; WBC, white blood cell.

White blood cell (WBC) function, production, and kinetics in horses are similar to those described in other species. Equine neutrophils circulate for approximately 10.5 hours and are equally distributed in the circulating and marginated neutrophil pools.⁷

GENERAL CONSIDERATIONS IN EQUINE LEUKOGRAM INTERPRETATION

Samples for complete blood counts (CBC) in horses should be handled with similar care as in other species. The preferred sample is EDTA-anticoagulated whole blood. Heparin anticoagulation is sufficient, but morphologic features and staining of cells are altered.⁴¹ Morphology of leukocytes is best if smears are made from EDTA-anticoagulated blood soon after collection. Refrigerated EDTA-anticoagulated blood samples maintain morphologic integrity fairly well for approximately 24 hours, but there are frequently clear cytoplasmic vacuoles, membrane irregularity, and ruptured

neutrophils seen on blood smears.¹⁷ If samples are processed after refrigeration of 48 hours or more, WBCs are typically severely smudged or unidentifiable on a stained blood smear. Analysis of equine blood by use of an Advia 120® hematology instrument found that artifactual leukocyte morphologic changes in blood stored for 24–48 hours included misclassification of granulocytes as mononuclear cells on the basophil channel. These changes were more pronounced in samples stored at 4°C compared with samples stored at 24°C.¹⁰

Neutrophil hypersegmentation (≥ 5 nuclear lobes) is an infrequent finding on equine blood smears, but can be seen in situations where there is delayed emigration of neutrophils from the vasculature into tissues (e.g. increased endogenous or exogenously administered corticosteroids). Neutrophil hyposegmentation is seen in band neutrophils in inflammatory conditions and in congenital Pelger-Huët anomaly, which has been reported in two Arabian horses (see Chapter 42).^{14,16,21}

If the WBC count is increased, 14,000–20,000 leukocytes per/ μL is considered moderate leukocytosis,

20,000–30,000 leukocytes/ μL is considered marked leukocytosis, and $>30,000$ leukocytes/ μL is considered extreme leukocytosis.¹⁷

Leukopenia is a significant finding and usually results from neutropenia because neutrophils are the predominant circulating WBC in healthy horses. Diseases that cause neutropenia are generally acute and severe. Neutropenia places the patient at increased risk for secondary bacterial infection.

Reference intervals determined by most laboratories are fairly wide because values from horses of different breeds, ages, and sexes are included.²⁴ Reference intervals for a specific breed of horse may have a much tighter interval. Differences between sexes are insignificant, but some age-related differences are important.

CELL MORPHOLOGY

The expected appearance of equine WBCs in Romanowsky-stained blood smears is described in Chapter 106.²² Segmented neutrophils have nearly colorless cytoplasm with a jagged, ribbon-shaped nucleus (Fig. 47.1). Band neutrophils frequently are slightly larger and have nearly parallel nuclear margins. The cytoplasm is colorless to pale blue.

Toxic neutrophils are typically large. Toxic changes affect the neutrophil nucleus and cytoplasm. They are caused by accelerated production and maturation during bone marrow development under the influences of various inflammatory mediators, cytokines, and products from infectious agents.¹⁵ Toxic changes of the cell and nucleus include overall cell swelling, nuclear swelling, reduced lobulation, and less condensation of chromatin. Toxic changes of the cytoplasm include increased basophilia (increased staining of RNA), vacuolization (from burst granules), Dohle bodies (lamellar stacks or whirls of rough endoplasmic reticulum that appear blue-gray), and toxic granulation (primary granules with increased permeability to stains that appear acidophilic to purple, Fig. 47.2).

Horses that have *Anaplasma phagocytophilum* (previous name *Ehrlichia equi*) infection may have neutrophils that contain morulae or mulberry-like inclusions in the cytoplasm (Fig. 47.3).²⁷ In contrast, inclusions have not been observed in WBCs from horses infected with *Ehrlichia risticii* (monocytic ehrlichiosis, Potomac horse fever, ehrlichial colitis).⁴⁴ Eosinophils are larger than neutrophils and have a lobulated nucleus with less condensed chromatin than neutrophils. Described as raspberry-like, eosinophils contain multiple, large, fairly uniformly sized, round, bright, red-orange granules (Fig. 47.4).

AGE, GENDER, AND EXERCISE RELATED LEUKOGRAM CHANGES

At birth, healthy foals have approximately 2.5 times more neutrophils than lymphocytes, variable numbers

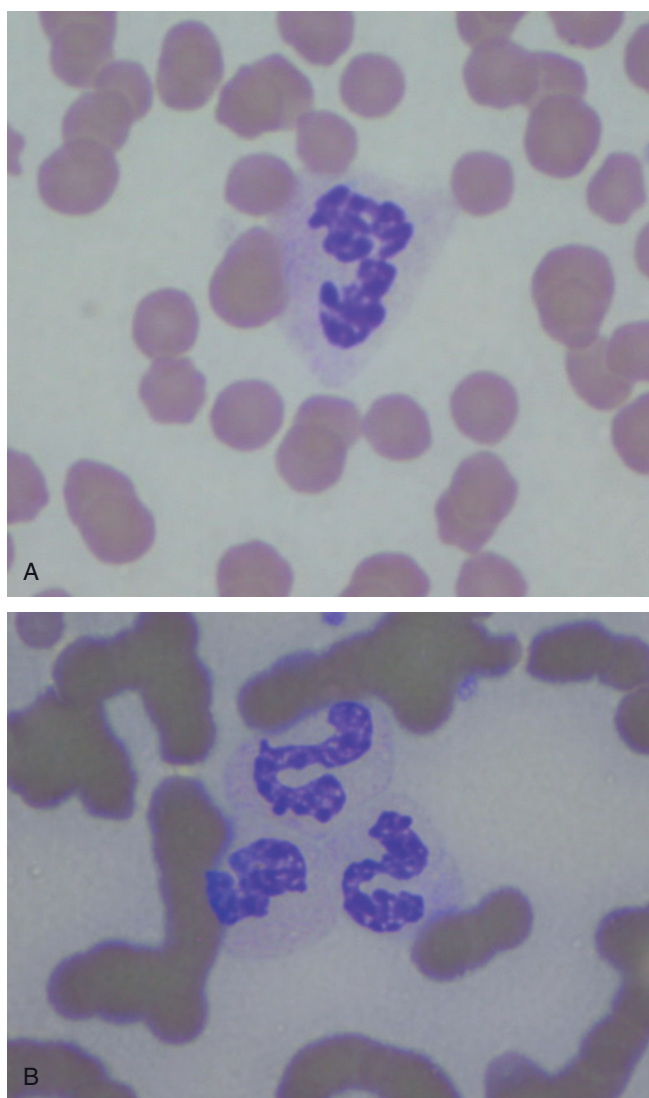


FIGURE 47.1 (A) Equine segmented neutrophil with distinct nuclear lobulation. (B) Three neutrophils that show typical jagged nuclear margins unlike the distinct nuclear lobulation in other species.

of monocytes, and an absence of eosinophils.¹⁷ Absolute neutrophil counts frequently exceed the reference interval for adult horses for several months, whereas lymphocyte counts are slightly less than the reference interval for adults. By 3–4 months of age, these WBC values reach adult reference intervals. Eosinophils gradually increase in number over several months, probably in response to intestinal parasite exposure. Premature foals have significantly lower neutrophil counts than term foals for the first several hours of life.⁹ Premature foals that survive may have nearly equal neutrophil and lymphocyte counts at birth, with the neutrophil proportion increasing rapidly within the first 18 hours of life. In contrast, the neutrophil-to-lymphocyte ratio does not increase significantly in

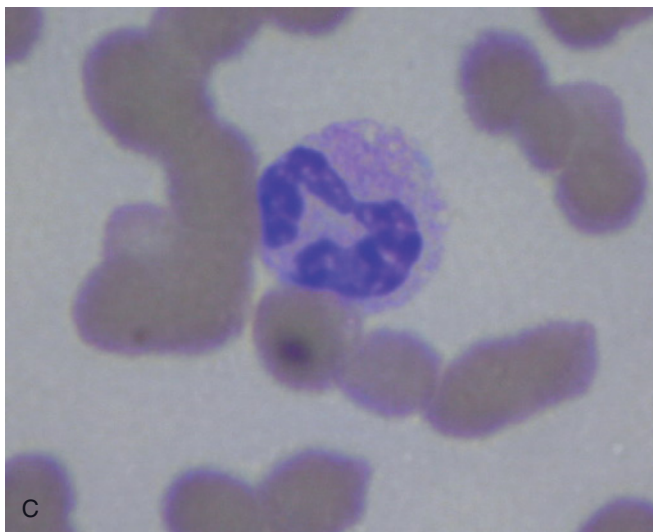
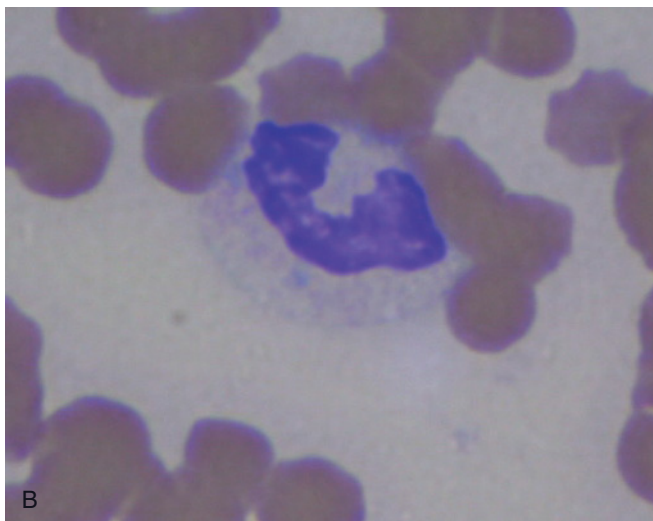
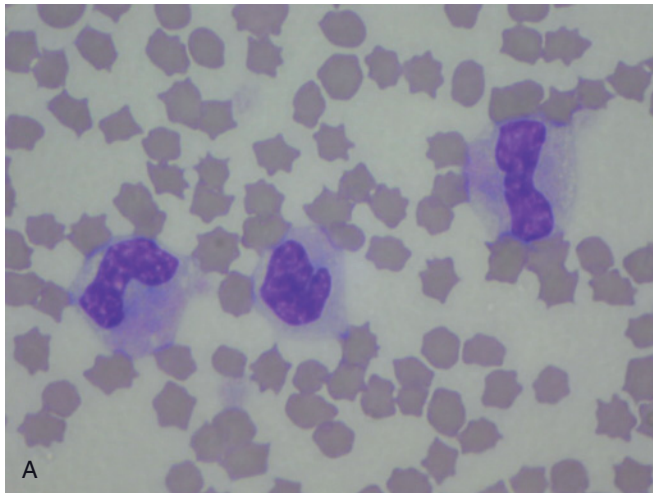


FIGURE 47.2 (A) Equine toxic neutrophils showing slightly swollen cells, swollen nuclei with hypolobulation, cytoplasmic basophilia, and cytoplasmic vacuolization. (B) Toxic band neutrophil showing cytoplasmic basophilia and a Dohle body. (C) Band neutrophil showing toxic granulation.

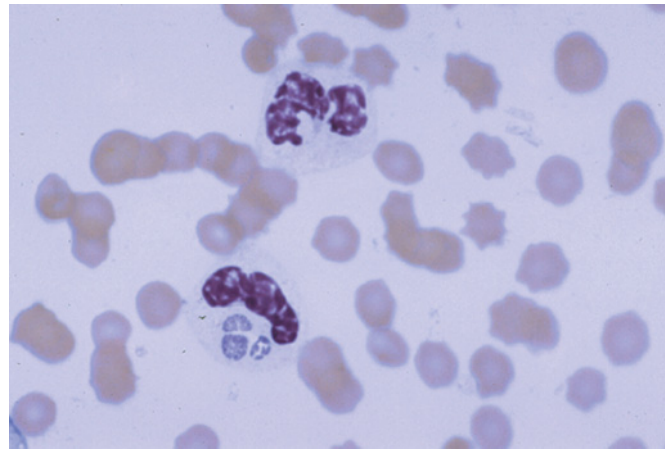


FIGURE 47.3 Morula of *Anaplasma phagocytophilum* in cytoplasm of a neutrophil in lower cell. (Courtesy of Ken Latimer, University of Georgia.)

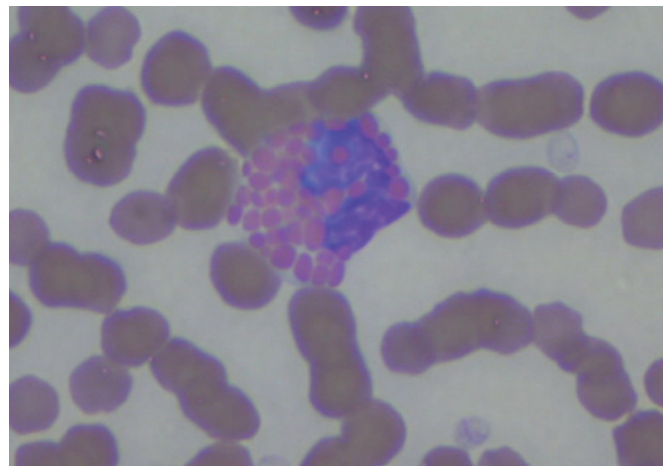


FIGURE 47.4 Equine eosinophil.

nonsurviving premature foals. However, these findings may not be evident before 35 hours after birth.

Total and differential WBC counts are similar in mares in the follicular and luteal phases of estrus.³⁶ Total WBC count is significantly decreased (but all values are within the typical reference intervals) in mares during pregnancy compared with postpartum samples owing to decreased T-helper and T-cytotoxic lymphocytes.¹

Granulocytes increase in circulation after prolonged strenuous exercise (e.g. endurance races and 3 day event competitions).^{2,37} Neutrophils are activated during intense exercise which results in degranulation.² Owing partially to increased levels of endogenous corticosteroids, neutrophil function is decreased for up to 3 days after prolonged strenuous activity (e.g. 80 km endurance race).³⁷

LEUKOGRAM RESPONSE PATTERNS

Although WBC types are kinetically independent and respond differently to various stimuli, they collectively demonstrate certain distinct response patterns.

Leukograms Characterized by Leukocytosis

Physiologic Leukocytosis

Physiologic leukocytosis³⁹ is a transient (20–30 minutes) but moderate increase in total WBC count, frequently reaching 12,000–25,000 cells/ μL . Typically, a modest mature neutrophilia exists in the absence of a left shift or toxic change. Lymphocytosis (6,000–14,000 lymphocytes/ μL) is frequently observed and may be proportionately greater than the increase in neutrophils.⁷ In frightening, painful, or exciting conditions, or after vigorous exercise activity, catecholamines increase cardiac output, hydrostatic blood pressure, and muscular activity. Neutrophils subsequently are swept from the marginated pool into the circulating pool, transiently increasing the numbers of cells enumerated by the WBC count. The causes of lymphocytosis are not as well documented; however, muscular activity may force cells in lymphatics into circulation and catecholamines may alter receptors on postcapillary venular endothelial cells and lymphocytes, blocking normal lymphocyte recirculating patterns.⁷ Horses have a marginated pool of neutrophils at least as large as the circulating pool; therefore, total cell counts may double with excitement. Physiologic leukocytosis is observed frequently in horses because they are easily excited, especially young hot-blooded horses and most horses that are unaccustomed to being handled.

Stress and Corticosteroid Effects

Stress and corticosteroid leukograms are characterized by a moderate neutrophilia, usually no left shift, lymphopenia, eosinopenia, and variable monocyte counts.^{7,8,33} This response is mediated by glucocorticoids of either endogenous or exogenous origin. Neutrophilia is caused predominately by early release of neutrophils from the bone marrow storage pool and to a lesser extent by decreased margination and emigration from the blood, causing neutrophils to circulate longer. Hypersegmented neutrophils may be observed on blood smears of some horses. Lymphopenia is caused by temporary sequestration of cells in lymphoid tissues. Lymphocytes and eosinophils generally are not killed at the concentrations of glucocorticoids obtained physiologically or achieved through most therapeutic uses of corticosteroids in horses. This response pattern may be seen in horses secondary to endogenous corticosteroid release in acute and severe diseases, with pituitary adenoma, or after administration of exogenous corticosteroids.

Inflammation/Infection-Associated Effects

Inflammatory changes in the leukogram have variable appearances depending on the severity and duration of the inciting agent, lesion, or condition.³⁸ A left shift is the hallmark of inflammation. Typically, these are band neutrophils, but less mature precursor cells also may be present. A significant left shift is present if there are greater than 300 bands/ μL , and the total WBC is within or greater than the reference interval. In the presence of neutropenia, a significant left shift exists if bands comprise at least 10% of the neutrophil population. If a neutropenia exists or the total WBC is within the reference interval and the number of immature neutrophils exceeds the number of mature segmented neutrophils, a degenerative left shift exists. Immature neutrophils and neutrophils demonstrating toxic changes have decreased functional ability compared with mature neutrophils.⁴⁰ Therefore, toxic bands or metamyelocytes may have significantly decreased functional capabilities compared with mature segmented neutrophils.

Toxic changes are graded on a subjective scale from +1 to +4 in most laboratories; the greater the number, the more severe the toxic change. If the cause of the condition is eliminated, the toxic changes disappear within 12–24 hours. The decrease or disappearance of toxic changes in neutrophils and an increasing neutrophil count are favorable prognostic signs.²⁷

Leukograms indicative of acute inflammation usually have a significant left shift with or without toxic changes in the neutrophils. Established or chronic inflammation may be difficult to recognize. Typically, leukocytosis is accompanied by mature neutrophilia because increased granulopoiesis over sufficient time (at least a week or more) has replenished the bone marrow storage pool such that only mature neutrophils are released into circulation and production has exceeded tissue usage. Additional information may be helpful for leukogram interpretation, including physical examination, history, previous leukogram data, and acute phase reactant levels. Serum acute phase reactant concentrations are useful mostly for assessment of inflammation, especially when the leukogram shows no or minimal indication of inflammation. Although rather insensitive, an increased concentration of fibrinogen or low (<10) plasma protein-to-fibrinogen ratio (which allows adjustments for alterations that are present because of dehydration and hemoconcentration) is indicative of inflammation. Another indicator of inflammation, that can be easily measured by automated instrumentation, is serum iron concentration. Hypoferremia is a more sensitive test than is increased fibrinogen concentration or low plasma protein-to-fibrinogen ratio.⁴ There is only one report of true iron deficiency anemia in horses; therefore, hypoferremia can be used as an indicator of inflammation.⁵

Eosinophilia

Eosinophilia is uncommon in horses and may be subtle when present. It usually is related to parasite or allergen

exposure,²⁸ however, some horses that have heavy intestinal parasite burdens have normal to low eosinophil counts.¹⁷ This may be related to the stress of disease inducing endogenous corticosteroid release. Horses may have significant tissue infiltration by eosinophils without a concurrent eosinophilia because of the short blood transit time and relatively long lifespan of eosinophils in tissues.³⁴ Cutaneous eosinophilic granulomas, either idiopathic or associated with parasite infection, may be accompanied by eosinophilia. Hypereosinophilic syndromes involving the gastrointestinal tract and pancreas or multiple organs have been reported intermittently in horses.²³ Cases of lymphoid leukemia or lymphosarcoma in horses have been reported with concurrent hypereosinophilic syndrome.²⁰ Such observations are similar to paraneoplastic syndromes in human patients in whom production of interleukin-5 (IL-5) by neoplastic T cells causes selective differentiation and activation of eosinophils.

Lymphocytosis

Lymphocytosis is a component of physiologic leukocytosis. Lymphocytosis may be observed infrequently after antigenic exposure and frequently accompanies lymphoid neoplasia.

Leukemias

Hematopoietic neoplasia that originates in the bone marrow is called leukemia (see Section V). Leukemias are rare in horses, but lymphocytic, granulocytic, monocytic, eosinophilic, and myelomonocytic leukemias have been reported.^{3,6,27,30,31} Lymphocytic leukemia is the most frequent leukemia in horses, followed by myelomonocytic leukemia. Total and differential leukocyte counts of the affected cell lines can be below, within, or above reference intervals. Abnormal or immature cells (blast cells) are frequently observed in stained blood smears. A preponderance of these cells is apparent in Romanowsky-stained bone marrow smears and aspirates or biopsies of other tissues and organs. Lymphoid leukemia has been reported as a primary disorder;² however, a leukemic blood picture also may occur with malignant lymphoma.²⁶ Lymphocyte counts may be within the reference interval, but abnormal cells may be observed on blood smears.⁴² Chronic lymphocytic leukemia has been diagnosed in two aged horses: one was a T cell type and the other was a B cell type with a monoclonal gammopathy (IgG) and light chain proteinuria.¹¹

Leukograms Characterized by Cytopenias

Lymphopenia

Lymphopenia is a predictable component of the stress-corticosteroid-associated leukogram pattern. In addition, lymphopenia may be observed with acute, severe inflammatory disorders and infections. In such condi-

tions, endogenous corticosteroid release is one cause. Furthermore, antigen-induced sequestration and trapping of cells in lymphoid tissues and organs occurs in response to infectious agents. In horses, lymphopenia is rarely caused by loss of lymphocyte-rich lymph into the thorax. Chylothorax has been diagnosed rarely in horses, but it has not been associated with lymphopenia. Combined immunodeficiency in Arabian and Arabian-cross foals is characterized by severe lymphopenia (<1000/mL) and lack of IgM production (see Chapter 57).^{25,35} A deficiency of both T and B cells exists. Hypoplasia of lymph nodes may be apparent in biopsy specimens. Alternatively, the presence of hypoplasia in other lymphoid tissues and organs can be documented more extensively during postmortem examination.

Monocytopenia, Eosinopenia, and Basopenia

Monocytopenia, if present, is not clinically significant. Eosinopenia may be part of a stress and corticosteroid leukogram. Basopenia is not clinically significant, if recognized at all.

Neutropenia

Neutropenia has significant clinical significance because it predisposes the patient to infection. Neutropenia can result from three major mechanisms. First, lack of or ineffective production of neutrophils may occur. Information from a group of eight related young Standardbred horses with significant neutropenia and intermittent thrombocytopenia has been reported.¹⁹ Bone marrow cultures from these horses revealed myeloid precursor cells were present and capable of responding to growth factors. Therefore, the investigators concluded that the cytopenias were caused by a familial bone marrow microenvironmental defect or a growth factor defect. Seven of the eight horses either died or were euthanized. Defective neutrophil production also has been associated with crowding of the marrow space (i.e. myelophthysis) by neoplastic cells (e.g. malignant lymphoma).³² Second, neutrophils can be rapidly redistributed from the circulation to the marginated pool after endotoxin exposure.¹³ Endotoxin causes an up-regulation of cell adhesion molecules on both neutrophils and endothelial cells.⁴³ If endotoxin exposure is terminated, a rebound neutrophilia occurs within 24 hours. However, if exposure is continued, prolonged neutropenia is the result of increased peripheral usage of cells. Third, increased peripheral usage or reduced survival of neutrophils may occur. In this most common cause of neutropenia, tissue demand for neutrophils exceeds the bone marrow's capacity to supply them. The transit time from bone marrow release to migration into tissues is usually shortened. Horses have approximately a 5 day supply of mature neutrophils in the bone marrow storage compartment in health, but this reserve can be rapidly depleted by demands of acute, severe infectious disease. Continued neutropenia with a left shift and toxic changes over several days in

TABLE 47.1 Serial Inflammatory Leukogram Findings and Their Interpretation

Leukogram Finding	Favorable Prognostic Sign	Unfavorable Prognostic Sign
Neutropenia	Increase in absolute neutrophil count	Continued neutropenia Development of greater left shift Development of toxic changes
Degenerative left shift	Increase in segmented neutrophils Decrease or lack of band neutrophils	Continued degenerative left shift
Toxic changes	Resolution of toxic changes	Continued toxic changes
Concurrent lymphopenia	Increase in absolute lymphocyte count	Continued lymphopenia

the face of appropriate therapy suggests a poor to grave prognosis.

Leukograms observed in foals or adult horses that have suspected septicemia or endotoxemia can have a variable appearance and must be interpreted in conjunction with other information collected from the patient. A moderate to marked leukopenia with left shift and toxic changes are leukogram findings highly consistent with septicemia or endotoxemia and are observed in most cases.^{18,29} At the time of admission to a hospital, foals with Gram-negative bacteremia are more likely to have lower total WBC and lymphocyte counts than foals with Gram-positive bacteremia. This may be helpful in the selection of antibiotics before obtaining bacterial culture results. Even with adequate passive transfer of immunoglobulin, most neonatal foals that have severe disease have leukopenia, neutropenia, and significant left shift with toxic changes in the neutrophils within 24 hours after hospital admission.¹²

Benefit of Serial Leukograms

Performance and interpretation of leukograms for two or more days are valuable in determination of the response to therapy, clinical prognosis, or confirmation of a diagnosis.^{18,27} Favorable and unfavorable prognostic signs related to data derived from serial leukograms are presented in Table 47.1.

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Interpretation of Canine Leukocyte Responses

A. ERIC SCHULTZE

Classical Canine Leukogram Patterns

- Physiologic Leukocytosis
- Corticosteroid-induced Leukocytosis
- Inflammatory Leukocytosis
 - Peracute inflammation
 - Acute inflammation
 - Chronic inflammation
 - Leukemoid reactions and extreme neutrophilia

Neutrophilia and Neutropenia

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- Causes and Mechanisms of Neutropenia
- Morphologic Alterations in Neutrophils
 - Nuclear alterations in neutrophils
 - Asynchronous nuclear maturation
 - Nuclear hypersegmentation
 - Nuclear hyposegmentation
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Infectious agents

Miscellaneous inclusions

- Eosinophilia and Eosinopenia
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- Basophilia and Basopenia
 - Causes and Mechanisms of Basophilia
 - Causes and Mechanisms of Basopenia
- Lymphocytosis and Lymphopenia
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 - Granular lymphocytes or natural killer cells
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- Monocytosis and Monocytopenia
 - Causes and Mechanisms of Monocytosis
 - Causes and Mechanisms of Monocytopenia
- Leukemia (see Section V)

Acronyms and Abbreviations

ACTH, adrenocorticotrophic hormone; EDTA, ethylenediaminetetraacetic acid; G-CSF, granulocyte colony-stimulating factor; WBC, white blood cell.

The total white blood cell (WBC) count in healthy dogs varies from 5,000 to 14,100 cells/ μ L of blood.³⁹ The neutrophil is the most commonly encountered leukocyte in the blood of healthy dogs. The half-life of circulating blood neutrophils is approximately 7.4 hours. Lymphocytes are less numerous than neutrophils. Monocytes and eosinophils are seen less frequently than neutrophils or lymphocytes, whereas basophils are seen rarely.

Total WBC counts are highest in puppies, and the counts gradually decrease throughout life. Both neutrophils and lymphocytes decrease in number with advancing age. Eosinophils also decrease in number but have minimal effect on the total leukocyte count.

Monocytes tend to increase slightly with advancing age.³¹

Diurnal variation in the total leukocyte count and absolute numbers of neutrophils, lymphocytes, and eosinophils can be detected in conditioned dogs kept under controlled light, feeding, and activity. The total WBC and absolute neutrophil counts reach maximum values in the late afternoon (7 pm). Lymphocyte counts are highest in late evening (11 pm) and lowest in the early morning (7 am). Eosinophil counts reach a zenith in late evening (11 pm) with the nadir at midday (12 pm). Although statistically different, diurnal variation has minimal effect on cell counts. Therefore, these daily cellular fluctuations would be unlikely to cause

problems in interpretation of clinical data from patients in which comparison is made to standard reference intervals.⁴²

CLASSICAL CANINE LEUKOGRAM PATTERNS

Physiologic Leukocytosis

Dogs that experience fear, excitement, or engage in brief periods of strenuous exercise may develop physiologic leukocytosis. This reaction is more common in puppies and is seldom seen in the adult dog. The leukogram is characterized by mild, mature neutrophilia and lymphocytosis. The monocyte and eosinophil counts may remain within the reference intervals or increase slightly. The magnitude of lymphocytosis may exceed that of the neutrophilia. Physiologic leukocytosis is transient, occurs within minutes of the stimulus, and usually resolves within 30 minutes. The mechanism of neutrophilia involves an epinephrine-mediated demargination of neutrophils from the marginal pool into the circulating pool, where they can be quantitated by the WBC count. Because the total number of neutrophils in the blood (sum of the marginal and circulating pools) does not change, this reaction is frequently termed pseudoneutrophilia. The lymphocytosis is believed to be caused by epinephrine-mediated blockade of lymphocytes entering lymphoid tissues or by mobilization of lymphocytes from the thoracic duct.^{31,40}

Corticosteroid-Induced Leukocytosis

Endogenous release of glucocorticoids because of severe stress or hyperadrenocorticism (Cushing's syndrome), and due to administration of exogenous glucocorticoids or adrenocorticotropic hormone may cause corticosteroid-induced leukocytosis in the dog. Total WBC counts usually range from 15,000 to 25,000/ μL of blood, but may occasionally reach 40,000/ μL of blood. The leukogram is characterized by mature neutrophilia, lymphopenia, monocytosis, and eosinopenia. Rarely, a mild left shift can occur if the bone marrow storage pool is depleted at the time of stimulation by corticosteroids. The leukogram alterations occur within 4–8 hours after a single administration of glucocorticoids and usually resolve within 24 hours. Leukograms from dogs given long-term corticosteroid therapy (>10 days) may require 2–3 days to return to baseline values after treatment ceases. The neutrophilia usually resolves before resolution of the lymphopenia. The mature neutrophilia is the result of several factors, including: decreased emigration of neutrophils from blood into tissues, increased release of mature neutrophils from the bone marrow storage pool, and decreased margination of neutrophils. The lymphopenia is attributed to redistribution of circulating lymphocytes. Long-term use of corticosteroids may cause lympholysis. The monocytosis may be the result of mobilization of mar-

ginated cells within the blood vasculature. Eosinopenia occurs because of inhibition of eosinophil release from the bone marrow and sequestration of eosinophils within tissues.^{31,40}

Inflammatory Leukocytosis

The leukogram is an excellent method by which to monitor inflammation in the dog. Infections caused by bacteria, rickettsia, viruses, fungi, or parasites cause alterations in the leukogram. Immune-mediated diseases, tissue necrosis, and neoplasia also elicit inflammatory reactions. Because neutrophils respond within hours to various chemotactic stimuli during inflammation, the time of phlebotomy relative to initial inflammatory stimulus is important in interpretation of the leukogram.

Peracute Inflammation

Sudden onset of an overwhelming infection or other inflammatory reaction can cause leukopenia characterized by neutropenia. Transient neutropenia can be detected within 1–3 hours after exposure to endotoxin. Overwhelming sepsis caused by infection of the lungs, thorax, uterus, or peritoneum by Gram-negative bacteria may cause similar responses. The neutropenia, which usually persists for 2–3 hours, is caused by endotoxin-mediated neutrophil margination within the vasculature, shortened neutrophil half-life, and increased cell emigration into tissues.^{31,40}

Acute Inflammation

Within 6–8 hours after an initial inflammatory stimulus, the bone marrow compensates by accelerated release of neutrophils, which results in neutrophilia. The acute inflammatory leukogram in the dog is characterized by leukocytosis. Total WBC counts usually range from 20,000 to 30,000/ μL of blood and are characterized by neutrophilia with a left shift (>450 bands, metamyelocytes, or myelocytes/ μL of blood). The neutrophilia and left shift occur because the production and early release of neutrophils by the bone marrow exceed the tissue demand for neutrophils. The left shift is usually orderly in that number of bands > metamyelocytes > myelocytes. Toxic changes may be observed in neutrophils. Concurrent lymphopenia and eosinopenia are typical owing to release of endogenous corticosteroids. Monocytosis is an inconsistent observation but may be present, particularly if a stress response is superimposed on the inflammatory leukogram.^{31,40}

Chronic Inflammation

Established inflammation of days to weeks duration can result in characteristic alterations in bone marrow, including expansion of the proliferative, maturation and, storage pools of neutrophils to meet tissue demand

TABLE 48.1 Causes of Leukemoid Reactions and Extreme Neutrophilia in Dogs

Infections/infectious agents	Chronic active peritonitis, <i>Hepatozoon canis</i> infection, internal abscesses, pyometra, salmon disease (lymphoid and monocytoid reaction)
Neoplasia	Metastatic fibrosarcoma, pulmonary adenocarcinoma, rectal adenomatous polyp, renal tubular carcinoma, renal tubular adenocarcinoma
Immune mediated	Immune-mediated hemolytic anemia
Other	Canine leukocyte adhesion deficiency (leukocyte glycoprotein CD11/CD18 deficiency), extreme eosinophilia from non-neoplastic causes (idiopathic hypereosinophilic syndrome)

for these cells. The leukogram is characterized by leukocytosis with neutrophilia; a left shift may be mild or absent. Alterations in the numbers of other leukocytes may be variable.

Leukemoid Reactions and Extreme Neutrophilia

The term leukemoid refers to a leukogram that resembles granulocytic leukemia in cell numbers and differential WBC counts but is caused by benign processes (Table 48.1). Typical leukemoid reactions are characterized by marked neutrophilic leukocytosis ranging from 50,000 to 100,000 cells/ μL of blood with a concurrent, orderly left shift that may extend to myelocytes or promyelocytes. Toxic change in neutrophils may or may not be present. Rarely, leukemoid reactions involve significant lymphocytosis or eosinophilia.^{1,33} Causes include chronic localized infections (e.g. pyometra, peritonitis), immune-mediated hemolytic anemia, CD11/CD18 neutrophil protein adhesion deficiency (canine granulocytopathy syndrome, canine leukocyte adhesion deficiency),²⁰ *Hepatozoon canis* infection, and paraneoplastic syndromes (e.g. renal tubular carcinoma,³⁵ metastatic fibrosarcoma,⁹ metastatic pulmonary adenocarcinoma,⁵⁹ and rectal adenomatous polyp⁵⁸). Terminology used by clinical pathologists for leukemoid reactions of extreme degree may vary. Leukograms characterized by neutrophilic leukocytosis (>100,000/ μL of blood) with a left shift and no evidence of hemopoietic neoplasia are sometimes referred to as extreme neutrophilic leukocytoses. The term leukoerythroblastic response has been used to describe a typical leukemoid reaction that is accompanied by metarubricytosis.⁴⁶ Differentiation of benign leukemoid reactions from chronic myelogenous leukemia can be challenging and frequently requires serial complete blood counts and bone marrow examinations. The diagnosis of chronic myelogenous leukemia is made by exclusion of causes of inflammation, presence of a disorderly left shift, or observation of abnormal granulocyte morphology in blood or bone marrow smears.

NEUTROPHILIA AND NEUTROPENIA

Causes and Mechanisms of Neutrophilia

Canine neutrophils have 2–5 nuclear lobes that are separated by constrictions. The nuclear outline is slightly irregular, and the chromatin is tightly condensed. The cytoplasm is moderate in amount and is filled with a myriad of granules that appear faintly pink in Romanowsky-stained blood films. The number of neutrophils in the blood of dogs depends on several factors, including rate of release from bone marrow, shifting of neutrophils between marginal pool and circulating pools in the blood, and emigration rate of neutrophils from blood into tissues.⁴⁰

Neutrophilia, defined as >12,000 neutrophils/ μL of blood, is a frequent observation in canine blood films (Table 48.2).⁴⁰ The more frequent causes of neutrophilia in dogs include physiologic leukocytosis, corticosteroid-induced leukocytosis, and inflammation. Additional causes of neutrophilia in dogs include hemolysis, hemorrhage, genetic defects in leukocyte adhesion molecules, and immune-mediated diseases. Several benign and malignant neoplastic conditions cause paraneoplastic syndromes that can result in neutrophilia. Thrombosis, infarction, burns, and uremia also can cause neutrophilia.^{31,40}

Inflammation is the most frequent cause of neutrophilia. The intensity of the underlying disease process has a direct effect on the magnitude of the total neutrophil response.³¹ Localized purulent lesions, like abscesses, induce greater neutrophilia than do more generalized diseases, such as septicemia. Neutrophilia with a left shift is the hallmark of acute inflammatory reactions. The degree of the left shift is considered a direct indication of severity of disease.³¹ Left shifts of great magnitude may occur in dogs that have pleuritis, peritonitis, pyoderma, or pyometra. Release of myelocytes and metamyelocytes from bone marrow is related to the increased tissue demand in purulent inflammation. However, in some diseases, inflammation is too mild to induce a left shift. Left shifts may be mild or nonexistent in seborrheic dermatitis, catarrhal enteritis, or hemorrhagic cystitis, conditions in which tissue demand for neutrophils is minimal.³¹

Causes and Mechanisms of Neutropenia

Neutropenia, defined as >2,900 neutrophils/ μL of blood, occurs less frequently than neutrophilia in dogs. Because neutrophils are the primary line of defense against microorganisms, neutropenia results in increased risk of infection. The more severe the degree of neutropenia, the greater the risk of infection. Three frequent mechanisms of neutropenia include decreased production of neutrophils in bone marrow, cellular shifting from the circulating to the marginal pool, and increased tissue emigration in excess of bone marrow release of neutrophils (Table 48.2).⁴⁰ Decreased production of neutrophils occurs from a variety of causes including dogs treated with total-body ionizing

TABLE 48.2 Causes of Neutrophilia and Neutropenia in Dogs**Neutrophilia**

Chemical and drug toxicity	Estrogen (acute phase only), recombinant granulocyte-CSF
Corticosteroid-induced leukocytosis	
Genetic adhesion molecule (CD11/CD18) deficiency	Canine leukocyte adhesion deficiency
Hemolysis	
Hemorrhage	
Immune-mediated diseases	Hemolytic anemia, systemic lupus erythematosus, polymyositis, polyserositis, rheumatoid arthritis, systemic necrotizing vasculitis
Infection	Bacteria (many species), viruses (canine distemper virus), fungi (many organisms), parasites (many species), rickettsia (Rocky Mountain spotted fever)
Inflammatory leukocytosis	Acute, chronic, or leukemoid responses
Leukemia	Several types, acute or chronic
Necrosis	Thrombosis, infarction, burns
Physiologic leukocytosis	
Tissue neoplasia/paraneoplastic syndromes	Several benign and malignant tumors
Toxemia	Endotoxemia, uremia

Neutropenia

Decreased production of neutrophils in bone marrow	
Bone marrow necrosis	
Cyclic hematopoiesis	
Drug administration	
Predictable	Gray collies (congenital), cyclophosphamide administration
Idiosyncratic	Estrogen toxicity (late), chemotherapeutic agents Cephalosporins, phenylbutazone, thiacetarsamide, Noxema ingestion Giant Schnauzers (congenital)
Malabsorption of vitamin B ₁₂	
Myelofibrosis and osteopetrosis	
Myeloproliferative and lymphoproliferative diseases	Several types of leukemia and lymphosarcoma
Radiation	
Cellular shifting from the circulating to the marginal pool	
Anaphylaxis	
Endotoxemia	
Increased tissue emigration in excess of bone marrow release	
Infection	Bacteria (many), rickettsia (ehrlichiosis), viruses (parvoviral enteritis, canine distemper virus), parasites (<i>Babesia canis</i>)
Immune-mediated disease	

irradiation, certain chemotherapeutic agents, and estrogen toxicosis (see Chapters 16–19, and 39). These conditions kill hemopoietic stem cells or progenitor cells or inhibit their replication. Significant neutropenia results from a single treatment of 12Gy in dogs that have total-body irradiation (see Chapter 39). Bone marrow transplantation or infusion of hemopoietic stem cells is necessary to repopulate the bone marrow and prevent death. Predictable neutropenia occurs in dogs treated with certain chemotherapeutic agents (see Chapter 16).³¹ The onset of neutropenia occurs within several days. Male dogs that have Sertoli cell tumors and those treated with estrogen for perianal gland tumors or prostatic hyperplasia may develop estrogen toxicosis (see Chapter 39).⁵⁷ Female dogs given diethylstilbestrol or estradiol cyclopentylpropionate for urinary incontinence, infertility, or mismating may also develop estrogen-induced bone marrow suppression (see Chapter 39).^{19,57} Idiosyncratic drug reactions (e.g. phenylbutazone, cephalosporin antibiotics, trimethoprim-

sulfadiazine, thiacetarsamide, phenobarbital) may cause neutropenia and pancytopenia in dogs (see Chapter 16).

The neutropenia and leukopenia of canine parvoviral enteritis can be attributed to several mechanisms (see Chapter 19). The virus is cytotoxic for hemopoietic stem cells. Endotoxemia resulting from gastrointestinal necrosis causes depletion of the maturation and storage pools of neutrophils in the bone marrow. Endotoxemia may cause increased margination of neutrophils in the vasculature as well as toxic injury to bone marrow.⁴⁹

Decreased production of neutrophils occurs in <30% of dogs that have chronic ehrlichiosis^{11,55} and in some Giant Schnauzer dogs that have inherited malabsorption of vitamin B₁₂ (see Chapters 30 and 31).¹⁷ Because of decreased hemopoietic space, bone marrow necrosis, myelofibrosis, and osteopetrosis result in neutropenia in dogs (see Chapters 17 and 18).^{27,64} Infrequently, disseminated granulomatous disease secondary to histoplasmosis¹⁰ and myelophthisis secondary to proliferation

of neoplastic cells within the marrow causes neutropenia in dogs (see Chapter 18).^{13,54} Myelophthisis has been associated with various leukemias and metastatic neoplasia in the bone marrow.

Cyclic hemopoiesis is an autosomal recessive disease of gray-collie dogs that has been attributed to mutation of the AP3B1 gene and altered intracellular transport of neutrophil elastase (see Chapter 42).²⁹ This stem-cell defect results in profound cyclic neutropenia. Untreated the disease is fatal within 3 years. Repeated endotoxin injections and administration of lithium carbonate control the neutropenia.⁷ Parenteral administration of recombinant granulocyte colony-stimulating factor (G-CSF) and lentivirus-mediated administration of G-CSF have also been used to treat this condition.⁶⁷ Long-term control of the disease requires bone marrow transplantation. Similar cyclic hemopoietic disease has been reported in cocker spaniels and Pomeranian dogs. Administration of cyclophosphamide also results in cyclic neutropenia.

A second mechanism of neutropenia is cellular shifting from the circulating to marginal pool. Rapid margination of neutrophils occurs with anaphylaxis and endotoxemia in dogs.

The third major mechanism of neutropenia is increased tissue emigration in excess of bone marrow release of neutrophils. Rapid use of neutrophils occurs with infections by pyogenic bacteria. Intense, acute, purulent infection of the lungs, uterus, gastrointestinal tract, or body cavities may cause moderate to significant neutropenia. A degenerative left shift, which occurs when the number of immature neutrophils is greater than that of mature neutrophils, may be seen in these conditions.⁴⁰ The total number of blood neutrophils may be normal or decreased.

While not a frequent finding, evidence for immune-mediated destruction of neutrophils in dogs is growing steadily. Weiss and colleagues have developed an indirect immunofluorescence assay to detect IgG binding to canine neutrophils and documented increasing neutrophil counts in affected dogs treated with immunosuppressive therapy.^{63,65}

Morphologic Alterations in Neutrophils

Nuclear Alterations in Neutrophils

Asynchronous Nuclear Maturation An indication of dysplasia, asynchronous nuclear maturation is characterized by a lobulated nucleus with an immature chromatin pattern. The neutrophil can appear slightly swollen and have some degree of toxic change. Nuclear lobes can be enlarged or coiled in unusual configurations, and the chromatin can appear dispersed. This uncommon nuclear alteration occurs in dogs that have significant resurgent neutrophilia, myeloid leukemia, and myelodysplastic or preleukemic syndromes.

Nuclear Hypersegmentation Neutrophils with >5 nuclear lobes are seldom observed in the blood of dogs (Fig. 48.1). Hypersegmented neutrophils are associated

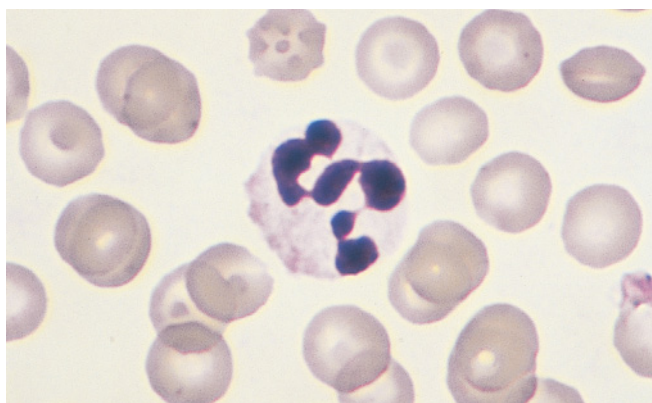


FIGURE 48.1 Hypersegmented neutrophil from a dog that had hyperadrenocorticism. Wright's stain.

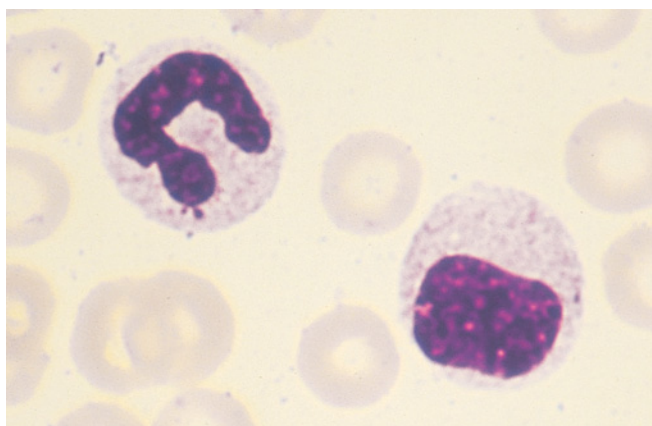


FIGURE 48.2 Late-band and metamyelocyte neutrophils from a dog that had inflammation. Wright's stain.

with prolonged transit times in the vasculature and occur secondary to severe stress (endogenous corticosteroid release), hyperadrenocorticism, and exogenous corticosteroid administration. Few normal to slightly larger, hypersegmented neutrophils are reported in toy and miniature Poodles that have congenital erythrocytic macrocytosis. Giant hypersegmented neutrophils have been observed in myelodysplastic syndromes and in granulocytic leukemia.^{31,39}

Nuclear Hyposegmentation Hyposegmentation of the neutrophil nucleus can be associated with the left shift of inflammation or infection, Pelger-Huët anomaly, or pseudo-Pelger-Huët anomaly. The key to differentiation between neutrophil immaturity (Fig. 48.2) and Pelger-Huët anomaly is assessment of the maturity of nuclear chromatin and observation of other immature neutrophilic cells in the blood smear that support a left shift. The chromatin in bands and metamyelocytes is progressively less condensed than that of neutrophils in Pelger-Huët anomaly in which there is a failure of the nucleus to segment.^{32,39}

Pelger-Huët Anomaly Reported in several breeds of dogs, Pelger-Huët anomaly is a benign condition



FIGURE 48.3 Neutrophil from dog with Pelger-Huët anomaly. Notice coarse mature chromatin pattern and hyposegmented nucleus. Wright's stain.

(Fig. 48.3). This hereditary disorder of granulocyte development is presumed to be transmitted as an autosomal dominant trait and is characterized by granulocytes and monocytes with hyposegmented nuclei and mature chromatin patterns. The nucleus of neutrophils, eosinophils, and basophils typically has a band or peanut-shaped nucleus with tightly condensed chromatin. Most dogs that have Pelger-Huët anomaly are heterozygous for the condition, and the neutrophils have normal function. Therefore, affected dogs have no predisposition for infection.^{5,36–38}

Cytoplasmic Alterations/Inclusions in Neutrophils

Ethylenediaminetetraacetic acid-induced Artifact Ethylenediaminetetraacetic acid (EDTA) induces artifacts in canine neutrophils when there is a delay in smear preparation.²¹ When whole blood is collected in EDTA and allowed to incubate at room temperature, neutrophils develop a few, clear vacuoles in the cytoplasm and an irregular distribution of cytoplasmic granules. Cell membranes appear irregular, and mild pyknosis may occur. Cytoplasmic basophilia and foamy vacuolation (i.e. signs of toxic change) are not observed as EDTA-induced artifactual change.

Toxic Change A set of disease-induced morphologic alterations in neutrophils, including cytoplasmic vacuolation, cytoplasmic basophilia, Döhle bodies, or prominently stained primary granules (toxic granulation) is referred to as a toxic change (Fig. 48.4).³⁹ These cytoplasmic lesions may be seen in cases of intense, localized or systemic infection, sterile inflammation, and drug toxicity.³¹

Cytoplasmic vacuolation occurs in neutrophils when bone marrow production of these cells is disrupted, resulting in loss of granule and membrane integrity. Persistent ribosomes impart the cytoplasm with its characteristic basophilia. Döhle bodies are angular, blue-to-gray, cytoplasmic inclusions that stain with variable intensity and measure approximately 0.5–2.0 μm in diameter. They represent lamellar aggregates

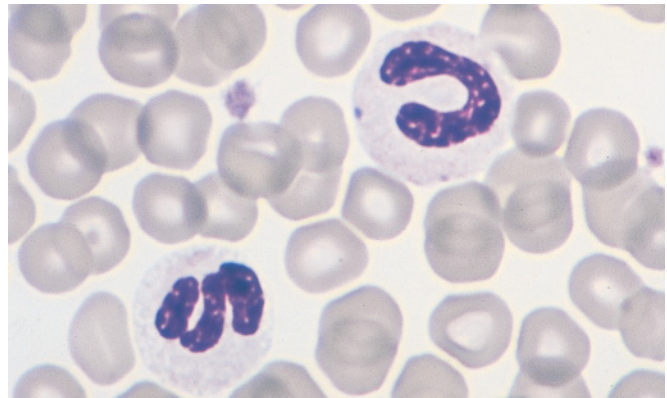


FIGURE 48.4 Canine segmented (left bottom) and band (right top) neutrophils with toxic change, characterized by cytoplasmic basophilia and Döhle bodies. Wright's stain.

of retained rough endoplasmic reticulum. Increased permeability of primary granule membranes to Romanowsky stains results in toxic granulation, a seldom seen change in canine neutrophils.^{22,23} Resolution of toxic change in neutrophils after appropriate treatment of the underlying disease is a favorable prognostic indicator in the dog.

Infectious Agents Bacterial rods and cocci can be infrequently detected in the cytoplasm of neutrophils and other blood phagocytes during septicemic crises.⁶⁰ The bacteria stain positively or negatively in Romanowsky-stained blood films and buffy coat preparations (Fig. 48.5). Although clinical signs and leukograms can provide supportive evidence for bacteremia, the diagnosis is best confirmed by blood culture.

Canine distemper virus inclusion bodies can be infrequently observed in neutrophils from dogs that have naturally occurring viral disease or post-vaccination (Fig. 48.6). The intracytoplasmic inclusions are round to irregularly shaped, homogeneous, magenta to gray-blue structures that may occur in blood cells, including erythrocytes and several types of leukocytes.³¹

Hepatozoon canis and *Hepatozoon americanum* gametocytes can be detected in the cytoplasm of neutrophils or monocytes. Parasitemia varies from high to low levels. It may be necessary to examine several blood films, capillary blood smears, or buffy coat preparations to identify gametocytes (Fig. 48.7). The oval gametocytes measure 5 \times 10 μm and are unstained to ice-blue within the cytoplasm of monocytes and neutrophils in Romanowsky-stained blood smears. Infection frequently is associated with neutrophilic leukocytosis.⁴⁴

Histoplasma capsulatum is a yeast-like fungus that can be observed in neutrophils, monocytes, and eosinophils in Romanowsky-stained blood films and buffy coats from dogs that have disseminated disease (Fig. 48.8). The organisms are round, 2–4 μm in diameter, and have thin walls. They have a small round, purple nucleus, and can occur singly or in clusters within the cytoplasm.¹⁰

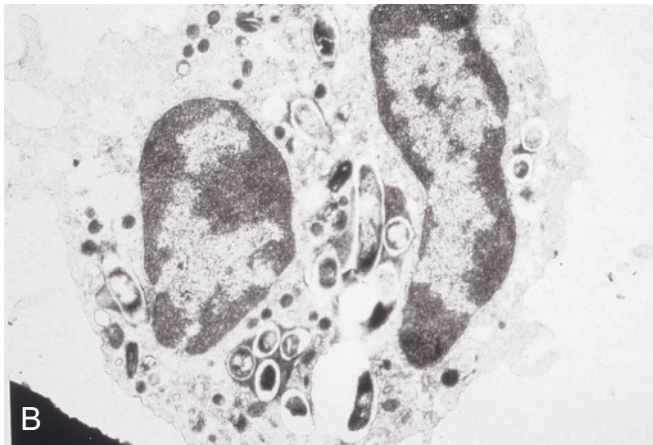
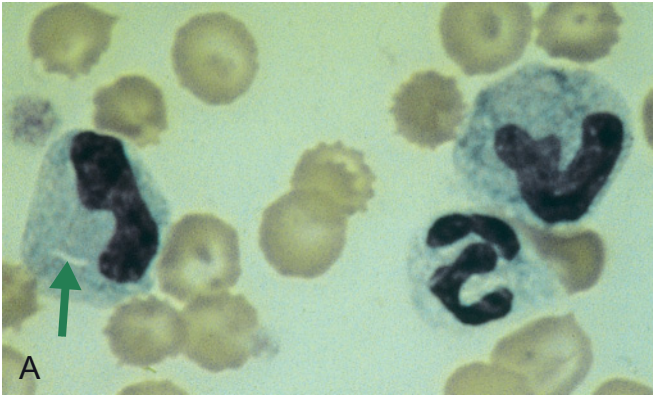


FIGURE 48.5 (A) Neutrophil with negatively stained intracellular bacillus (arrow) in blood smear from a dog that has mycobacteriosis, Wright's stain. (B) Transmission electron micrograph of a neutrophil from the same dog that has several bacilli. (Courtesy of Dr. Harold W. Tvedten, Michigan State University, East Lansing, MI.)

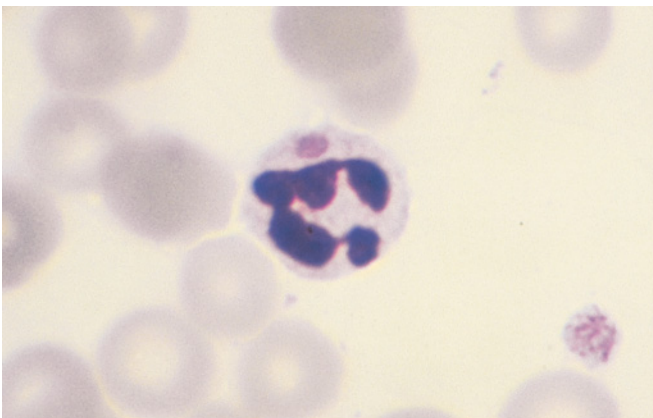


FIGURE 48.6 Magenta-colored canine distemper virus inclusion in a neutrophil, Wright's stain.

Leishmania donovani amastigotes rarely have been identified in neutrophils from dogs that have disseminated leishmaniasis.⁵³ Leukocytosis with neutrophilic left shift is a frequent finding. The amastigotes are small, round to oval organisms and occur 1–2 organ-

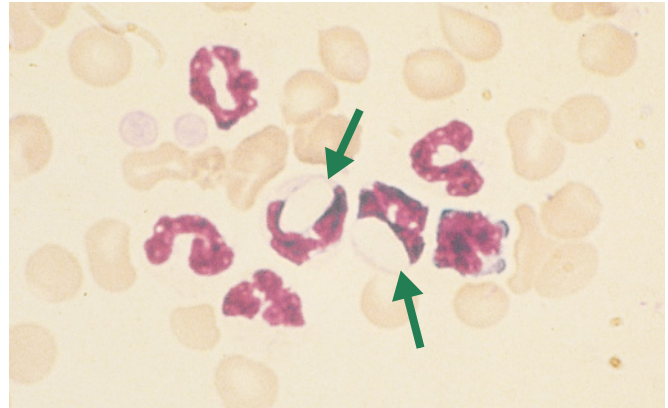


FIGURE 48.7 Two segmented neutrophils with intracytoplasmic, clear to ice-blue *H. canis* gametocytes. Wright's stain. (Courtesy of Dr. Robert Green, Texas A&M University, College Station, TX.)

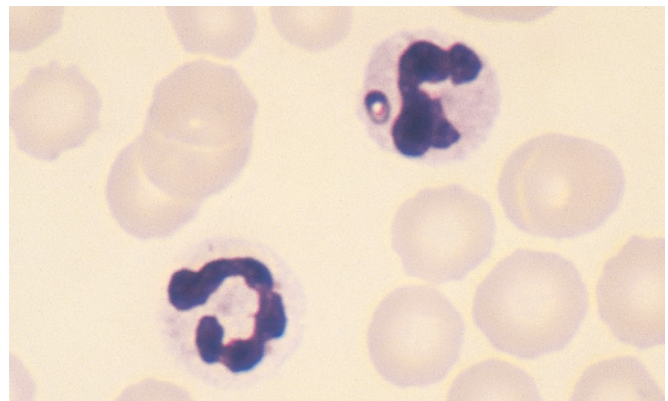


FIGURE 48.8 Segmented neutrophil with intracytoplasmic *Histoplasma capsulatum*. Wright's stain.

isms per neutrophil. They have an oval nucleus, basophilic ventral kinetoplast, and light blue cytoplasm. Amastigotes have been identified in approximately 3% of circulating neutrophils.

Anaplasma phagocytophilum morulae are identified frequently in neutrophils from infected dogs and *Ehrlichia canis* morulae can be observed infrequently in monocytes.⁵⁶ Morulae from the different strains of *Ehrlichia* species appear similar. The *Ehrlichia* morulae vary from magenta to blue-gray in color and resemble a mulberry (Fig. 48.9). The identity of the specific agent can be established by polymerase chain reaction, RNA or DNA sequencing, or by determination of acute and convalescent serum antibody titers.

Miscellaneous Inclusions Hemosiderin granules have been observed in the cytoplasm of occasional neutrophils and monocytes from dogs with immune-mediated hemolytic anemia after transfusion therapy. The brown granules, that stain positive for iron, measured 1–4 μm in diameter (Fig. 48.10). Multiple granules can be observed in some cells. The mechanism of their occurrence in neutrophils is uncertain.¹⁸

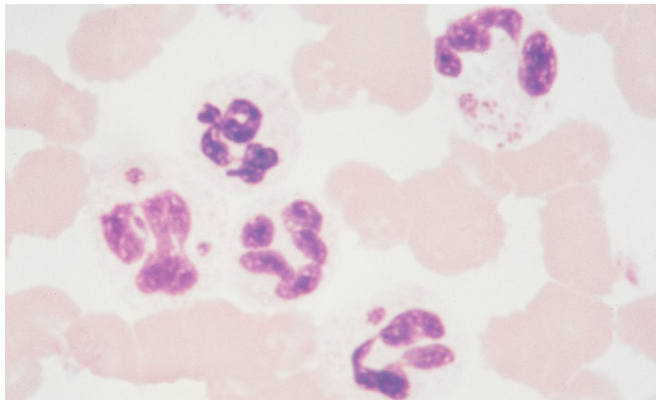


FIGURE 48.9 Three neutrophils containing morulae of *Ehrlichia ewingii*, Wright's stain. (Courtesy of Dr. Steven L. Stockham, Kansas State University, Manhattan, KS.)

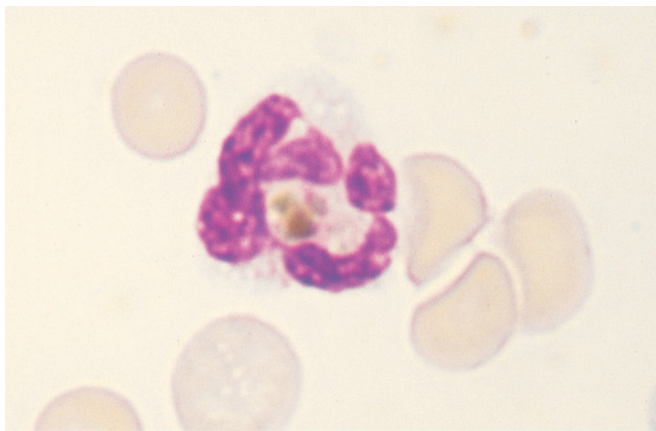


FIGURE 48.10 Segmented neutrophil with hemosiderin crystal from a dog that had immune-mediated hemolytic anemia. Wright's stain. (Courtesy of Dr. Stephen Gaunt, Louisiana State University, Baton Rouge, LA.)

Mucopolysaccharidosis type VI (arylsulfatase B deficiency) and type VII (β -glucuronidase deficiency) are lysosomal storage diseases that have been reported rarely in the dog. Neutrophils and lymphocytes from affected dogs can have large, pink-to-purple staining inclusions (Alder-Reilly bodies) in the cytoplasm in routine Romanowsky-stained blood films (Fig. 48.11). The inclusions in mucopolysaccharidosis type VII can stain metachromatically with 1% toluidine blue dye. The granules represent intermediate products that accumulate in cells caused by the arylsulfatase B (type VI) or β -glucuronidase deficiency (type VII). Disease inheritance is by means of an autosomal recessive pattern.^{26,47}

EOSINOPHILIA AND EOSINOPENIA

Causes and Mechanisms of Eosinophilia

In Romanowsky-stained blood films, canine eosinophils have a bilobed or trilobed nucleus with condensed chromatin and abundant cytoplasm that contains

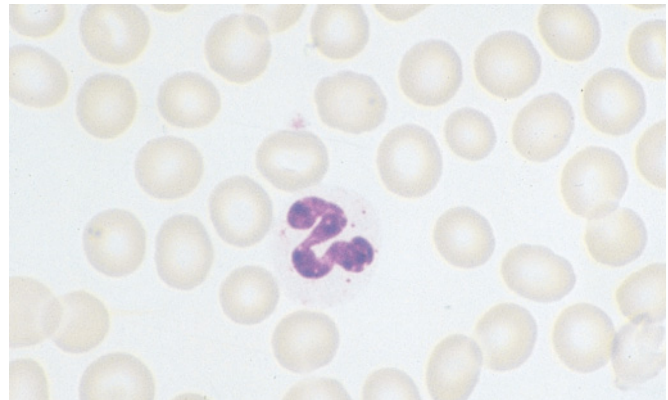


FIGURE 48.11 Segmented neutrophil with Alder-Reilly bodies from a dog that had mucopolysaccharidosis type VII. Wright's stain. (Courtesy of Dr. Mary Anna Thrall, Colorado State University, Ft. Collins, CO and Dr. Mark Haskins, University of Pennsylvania, Philadelphia, PA.)

round, red-orange granules. The granules frequently vary in number and size. In some dogs, eosinophils appear degranulated or vacuolated. The primary functions of eosinophils are to destroy parasites (primarily helminths) and to modulate hypersensitivity reactions (see Chapter 43).

Eosinophilia, defined as $>1,300$ eosinophils/ μL of blood, is a frequent occurrence in dogs (Table 48.3).³⁹ The most common cause of eosinophilia in dogs is parasitism. Both endoparasites and ectoparasites cause eosinophilia. However, this alteration occurs more often and is of greater magnitude with endoparasites that migrate through body tissues and have prolonged host-tissue contact. Inflammation or local hypersensitivity reactions in the alimentary, respiratory, and genitourinary systems and in skin that are mediated by mast cell degranulation also cause eosinophilia. In some dogs, localized tissue eosinophilia may occur in the presence of normal blood eosinophil counts.³¹

Eosinophilia may occur as a paraneoplastic syndrome in some dogs.^{12,43,50} Although many malignant and some benign neoplasms have been reported to cause eosinophilia, disseminated mast cell neoplasia most frequently causes this alteration in dogs. The mechanism of eosinophilia is unknown. However, T lymphocyte-derived cytokine mediators, such as interleukin-2 and -5, are probably involved.³⁶ Idiopathic hypereosinophilic syndrome occurs rarely in dogs and is characterized by persistent eosinophilia with extensive tissue infiltration and organ dysfunction of unknown cause (see Chapter 43).² It may be difficult to differentiate hypereosinophilic syndrome from eosinophilic leukemia. Rarely, eosinophilia is reported after administration of certain drugs in the dog. Eosinophilia has been associated with tetracycline and recombinant interleukin-2 administration.^{6,14}

Causes and Mechanisms of Eosinopenia

Eosinophil counts in healthy dogs range from 0 to 1,300 eosinophils/ μL of blood.³⁹ Because many normal dogs

TABLE 48.3 Causes of Eosinophilia and Eosinopenia in Dogs

Eosinophilia	
Drug administration	Recombinant interleukin-2 administration, tetracycline
Hypoadrenocorticism	
Hypersensitivity reactions (alimentary, genitourinary, respiratory systems, skin and special senses may be affected)	Oral granuloma, ulcerative/eosinophilic gastroenteritis, gastrointestinal eosinophilic granuloma, pyometra, myositis, panosteitis, pulmonary infiltrates with eosinophilia, canine eosinophilic granuloma, sterile eosinophilic pustulosis
Fungi/yeastlike fungi	<i>Aspergillus fumigatus</i> , <i>Cryptococcus neoformans</i> , <i>Pythium insidiosum</i>
Parasites	
Nematodes	<i>Ancylostoma</i> sp., <i>Angiostrongylus vasorum</i> , <i>Ascarids</i> , <i>Dipetalonema reconditum</i> , <i>Dirofilaria immitis</i> , <i>Oslerus (Filaroides) osleri</i> , <i>Filaroides hirthei</i> , <i>Physaloptera</i> sp., <i>Spirocerca lupi</i> , <i>Trichinella spiralis</i> , <i>Trichuris vulpis</i>
Trematodes	<i>Heterobilharzia americana</i> , <i>Paragonimus kellicotti</i> , <i>Alaria</i> sp.
Arthropods	<i>Dermatobia hominis</i> , fleas, ticks, <i>Sarcoptes scabiei</i> var. <i>canis</i> , pentastomes
Protozoa	<i>Babesia canis</i> , <i>Hepatozoon canis</i> , <i>Pneumocystis carinii</i> , <i>Sarcocystis canis</i> , <i>Haemobatonella canis</i>
Neoplasia	Idiopathic hypereosinophilic syndrome, myeloid leukemia, paraneoplastic syndromes (fibrosarcoma, lymphomatoid granulomatosis, mammary carcinoma, disseminated mastocytosis, rectal polyp, thymoma, T-cell lymphoma), essential thrombocythemia
Eosinopenia	
Corticosteroids	
Endogenous	Hyperadrenocorticism, stress
Exogenous	Corticosteroid treatment
Acute infection/inflammation	

may routinely have eosinophil counts of 0/ μL , true eosinopenia is difficult to document and is of limited clinical significance. Determination of absolute eosinophil counts with a hemocytometer and eosin-based diluent is recommended to confirm suspected cases of eosinopenia and may be valuable in monitoring responses to *o,p'*-DDD (mitotane) treatment in dogs that have hyperadrenocorticism.³¹

Eosinopenia may occur in dogs that have emotional or physical stress, or have inflammatory disease owing to release of adrenocorticosteroids as well, as in hyperadrenocorticism (Cushing's syndrome).⁴⁰ It is hypothesized that corticosteroids inhibit histamine release, neutralize circulating histamine, and initiate release of cytokines that mediate alterations in eosinophil distribution. Eosinopenia from a single dose of corticosteroids occurs within 1–6 hours and counts return to normal within 12–24 hours. Long-term use of high-dose corticosteroids depresses eosinophil production by the bone marrow.⁴⁰

BASOPHILIA AND BASOPENIA

Causes and Mechanisms of Basophilia

Basophils are seen infrequently in blood films submitted for hematologic evaluation. These unique cells, which are slightly larger than segmented neutrophils, usually account for <2% of the differential leukocyte count or an absolute count of 0–140 basophils/ μL .³⁹ Basophils have a poorly lobulated nucleus that has been described as a twisted ribbon (see Chapter 104). The

blue-gray cytoplasm contains widely scattered, round, metachromatic granules that vary greatly in size and number.

Basophil granules contain preformed mediators, including histamine and heparin (see Chapter 44). When stimulated, these cells synthesize platelet-activating factor; thromboxane A₂; and leukotrienes C₄, D₄, and E₄.³² Basophils are intricately involved in host defense by means of immunoglobulin E (IgE)-mediated inflammatory reactions (see Chapter 44).^{25,30}

Basophilia, defined as a prolonged increase in circulating numbers of basophils (>140 basophils/ μL of blood), is a rare event. Because numbers of circulating basophils are low in health, the routine differential cell count is a rather insensitive measure of basophil numbers. Only sustained increases in basophil numbers of >3–6% of the differential cell count can be detected on routine blood smears.³⁶ Usually, basophilia accompanies eosinophilia but can occur independently. The most frequent cause of basophilia in the dog is infection with the canine heartworm, *Dirofilaria immitis* (Table 48.4).³⁶ Basophilia also is associated with infection with several other parasites and infectious agents. In addition, basophilia is associated with various hypersensitivity or inflammatory conditions, neoplastic diseases, and as an infrequent consequence of the administration of certain drugs.^{36,48} Although it has been suggested that basophilia is a result of lipemia associated with chronic liver disease, nephrotic syndrome, and endocrine disturbances, including diabetes mellitus and hyperadrenocorticism, these assertions have not been confirmed in many cases. Basophilia caused by basophilic leukemia is rare.⁴⁵

Causes and Mechanisms of Basopenia

Basophil counts in healthy dogs range from 0 to 140 basophils/ μL .³⁹ Because many normal dogs routinely have basophil counts of 0/ μL , basopenia is difficult to document with currently available methods and is considered clinically insignificant at this time.

TABLE 48.4 Causes of Basophilia in Dogs

Drug administration	Heparin, penicillin
Hypersensitivity and/or inflammatory lesions	Allergic respiratory disease, cutaneous eosinophilic granuloma, eosinophilic gastroenteritis, experimental <i>Candida crusei</i> and <i>Candida albicans</i> administration, osteomyelitis, pulmonary eosinophilic granuloma, pulmonary infiltrates with eosinophilia
Neoplasia	Basophilic leukemia, disseminated mast cell neoplasia, essential thrombocythemia, lymphomatoid granulomatosis, thymoma
Parasitic diseases	<i>Ancylostoma</i> and <i>Uncinaria</i> sp., <i>Dirofilaria immitis</i> , <i>Dipetalonema reconditum</i> , <i>Hepatozoon canis</i> , <i>Crenosoma vulpis</i>

LYMPHOCYTOSIS AND LYMPHOPENIA

Causes and Mechanisms of Lymphocytosis

Canine peripheral blood lymphocytes are predominately small, round mononuclear cells (see Chapter 104). They have a round to oval nucleus with aggregated chromatin and have indistinct nucleoli. The blue cytoplasm is scant and may contain a few azurophilic granules. In healthy dogs, the lymphocyte is the second most frequently encountered WBC. B and T cells appear similar by light microscopic examination of Romanowsky-stained blood films. However, the majority of lymphocytes in the blood are T cells.³⁹ In general, B cells are short-lived (days to weeks) compared with T cells (months to years). Lymphocytes have the capacity to recirculate and undergo mitosis.³¹

Lymphocytosis, defined as $>2,900$ lymphocytes/ μL of blood, is an uncommon occurrence in the dog (Table 48.5). Physiologic leukocytosis may cause a transient lymphocytosis. Physiologic lymphocytosis does not occur frequently in the adult dog but occurs more frequently in puppies. This form of lymphocytosis can be avoided if animals are not overly excited at time of venipuncture. Examination of a second sample of blood, collected from the calmed or tranquilized dog, can be used to distinguish transient lymphocytosis of

TABLE 48.5 Causes of Lymphocytosis and Lymphopenia in Dogs

Lymphocytosis

Chronic antigenic stimulation	Aspergillosis, actinomyces, <i>Babesia canis</i> infection, blastomycosis, brucellosis, ehrlichiosis, encephalitozoonosis, leishmaniasis, pneumocystis pneumonia, Rocky Mountain spotted fever, <i>Trypanosoma cruzi gambiense</i> infection
Hypoadrenocorticism	
Lymphoid neoplasia	Lymphocytic leukemia (acute or chronic), lymphosarcoma, thymoma
Physiologic leukocytosis (not common in the dog)	

Lymphopenia

Acute systemic bacterial infections	Septicemia, endotoxemia
Corticosteroids	Stress-induced leukocytosis (pain, extremes in body temperature), hyperadrenocorticism (Cushing's syndrome), exogenous corticosteroid therapy or ACTH administration
Disruption of lymph node architecture	Generalized granulomatous disease, multicentric lymphosarcoma
Immunodeficiency syndromes	Combined T- and B-cell deficiency of Basset hounds, combined immunodeficiency of Jack Russell terriers
Immunosuppressive drugs	
Loss of lymphocyte rich fluids	Protein-losing enteropathy (lymphangiectasia), ulcerative enteritis, granulomatous enteritis, chylothorax, chyloperitoneum
Malignant neoplasia	Lymphosarcoma, lymphocytic leukemia
Radiation	
Viral infections (acute stages usually)	Canine distemper, infectious canine hepatitis, coronavirus enteritis, canine parvovirus

physiologic leukocytosis from lymphocytosis of pathologic origin.³⁶

Persistent antigenic stimulation in chronic infections or inflammatory reactions may cause lymphocytosis in dogs. Chronic canine ehrlichiosis¹¹ and Rocky Mountain spotted fever are diseases that result in proliferation of lymphocytes and expansion of the blood lymphocyte pool. Trypanosomiasis,³ leishmaniasis,²⁴ and brucellosis⁶¹ also may cause lymphocytosis by a similar mechanism.

Lymphoid neoplasia can cause lymphocytosis.⁵¹ Malignant lymphoma, acute or chronic lymphocytic leukemia, and thymoma have been associated with lymphocytosis (see Chapters 69 and 77). The highest lymphocyte counts usually are associated with chronic lymphocytic leukemia.⁴¹ Malignant lymphoma, a neoplastic proliferation of lymphocytes within tissue, also can produce lymphocytosis in approximately 10% of dogs that have this disease.

Hypoadrenocorticism (Addison's disease) has been reported to cause lymphocytosis in 11–20% of affected dogs.⁶⁶ The lack of lymphopenia in a severely stressed dog provides supportive evidence for glucocorticoid deficiency.

Causes and Mechanisms of Lymphopenia

Lymphopenia, defined as <400 lymphocytes/ μ L blood, is a frequent occurrence in dogs and may result from several mechanisms (Table 48.5).³⁹ Physical stresses such as extremes in temperature or pain, which result in excess corticosteroid release, cause lymphopenia of transient and predictable nature. Exogenous administration of corticosteroids or ACTH and hyperadrenocorticism also cause lymphopenia. Acute infections can cause lymphopenia by means of stress-induced corticosteroid release and redistribution of lymphocytes.^{31,36} Specific antigens can cause lymphocytes to become trapped in lymphoid tissues. Lymphopenia can occur when lymphocytes are recruited to antigenically stimulated lymph nodes and then emigrate into tissues.³⁶ Inflamed lymph nodes can occlude efferent lymph flow, thus preventing lymphocyte recirculation and compounding lymphopenia.²⁸ Certain viruses, particularly canine distemper virus and canine parvovirus, cause lymphocyte destruction, atrophy of lymphoid tissues, and depletion of lymphocyte subpopulations.³⁶

Some cases of protein-losing enteropathy, lymphangiectasia, ulcerative enteritis, and granulomatous enteritis in dogs cause lymphopenia as a result of loss of lymphocyte-rich fluid into the intestinal lumen.³⁴ Chylothorax and chyloperitoneum can result in lymphopenia due to sequestration of lymphocyte-rich fluids in body cavities.¹⁶ Repeated centesis and removal of chyle from body cavities may exacerbate lymphopenia. Occlusion of the flow of lymph caused by disseminated granulomatous inflammation or neoplasia also can result in lymphopenia.

Rarely, lymphopenia can be the result of congenital lymphocyte deficiency.¹⁵ Basset hounds that have combined immunodeficiency have lymphopenia and recur-

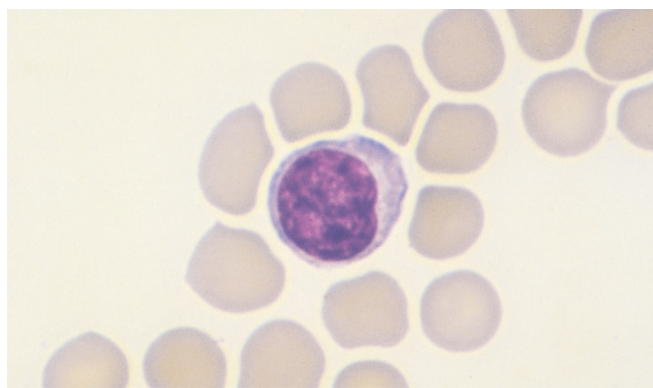


FIGURE 48.12 Reactive lymphocyte in a canine blood smear. Wright's stain.

rent infections such as mycobacteriosis (see Chapter 57).⁸ Autosomal recessive severe combined immunodeficiency of Jack Russell terriers results in puppies with marked lymphopenia and decreases in the concentrations of serum immunoglobulins secondary to hypoplasia of all lymphoid tissues.⁴

Morphologic Alterations in Lymphocytes

Reactive Lymphocytes

Reactive lymphocytes (also termed immunocytes and transformed lymphocytes) are antigenically stimulated lymphoid cells that are seen in the blood of dogs (Fig. 48.12). These cells have a deeply basophilic cytoplasm. Reactive lymphocytes vary in size, nuclear chromatin clumping, and number of nucleoli visualized. While some resemble small lymphocytes with a basophilic cytoplasm, others resemble lymphoblasts. The cytoplasm may have a perinuclear Golgi zone and may contain a few vacuoles and resemble plasma cells.^{36,39}

Plasma Cells

Plasma cells are rare in the blood of dogs and are usually observed in bone marrow or lymph-node aspirates (Fig. 48.13). They are large cells with intensely basophilic cytoplasm and a prominent, pale-staining, perinuclear Golgi zone. The nucleus is usually eccentrically located and has condensed chromatin.^{36,39}

Granular Lymphocytes or Natural Killer Cells

Granular lymphocytes or natural killer cells are null lymphoid cells that are seen infrequently in blood smears (Fig. 48.14). They have a few distinct azurophilic granules that tend to cluster at the nuclear margin or indentation.³⁶

Lymphoblasts

Lymphoblasts are large lymphoid cells that are observed frequently in disseminated lymphoma and

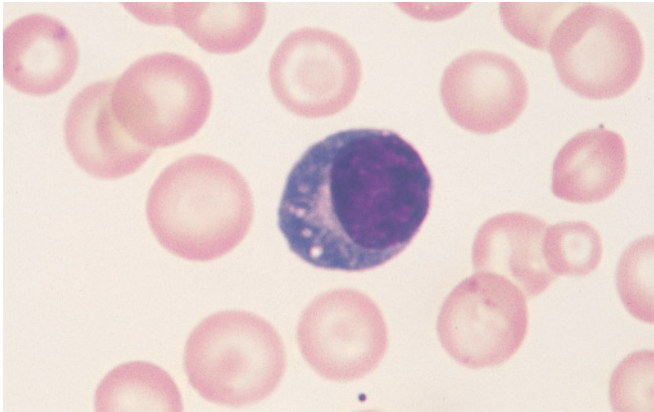


FIGURE 48.13 Plasma cell in canine blood smear. Wright's stain. (Courtesy of Dr. Steven L. Stockham, Kansas State University, Manhattan, KS.)

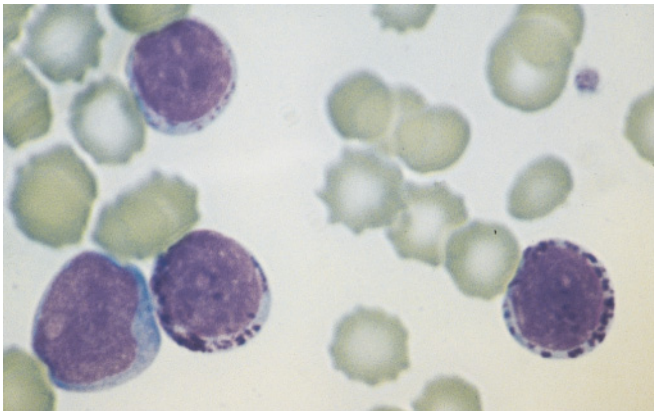


FIGURE 48.14 Large granular lymphocytes in a canine blood smear. Wright's stain. (Courtesy of Dr. Maxey L. Wellman, The Ohio State University, Columbus, OH.)

lymphoblastic leukemia (Fig. 48.15). Lymphoblasts have a large nucleus with vesicular chromatin and prominent nucleoli. The cytoplasm is abundant and has a deep blue hue.^{36,39}

Phospholipidosis-Induced Cytoplasmic Vacuoles

Lymphocytes from dogs with drug-induced phospholipidosis may have large clear cytoplasmic vacuoles.⁶² These vacuoles occur singly or may be multiple and may push the nucleus eccentrically. Affected lymphocytes are readily visible upon examination of Wright-stained blood smears.⁵²

MONOCYTOSIS AND MONOCYTOPENIA

Causes and Mechanisms of Monocytosis

Canine monocytes are large cells with pleomorphic (round, oval, bilobed, or multilobed) nuclei and fine lacy nuclear chromatin patterns (see Chapter 104). Monocytes have large amounts of blue-gray cytoplasm

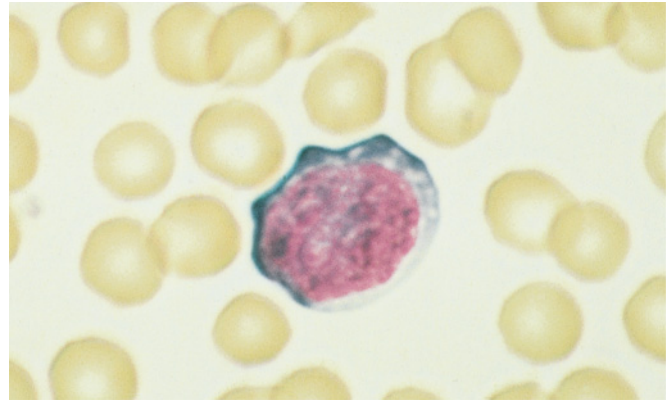


FIGURE 48.15 Lymphoblast in blood smear of a dog that has malignant lymphoma. Notice the prominent nucleoli. Wright's stain. (Courtesy of Dr. Rose Raskin, Purdue University, West Lafayette, IN.)

TABLE 48.6 Causes of Monocytosis in Dogs

Inflammatory lesions (acute or chronic)	Bacteremia/septicemia, bacterial endocarditis, immune-mediated injury (hemolytic anemia), necrosis/suppurative (tissue or body cavities), osteomyelitis, pyogranulomatous disease (several causes)
Miscellaneous causes	Corticosteroid-induced leukocytosis, hemorrhage/hemolysis, rebound neutropenia/cyclic hematopoiesis, recombinant G-CSF administration, trauma
Neoplasia	Benign tumors (rectal polyp), malignant tumors (chondrosarcoma, hemangiosarcoma, lymphosarcoma), malignant histiocytosis, monocytic leukemia, myelomonocytic leukemia
Parasitic diseases	<i>Hepatozoon canis</i> infection, <i>Pneumocystis carinii</i> pneumonia, <i>Trypanosoma brucei</i> infection, <i>Angiostrongylus vasorum</i> infection, <i>Crenosoma vulpis</i> infection, <i>Dirofilaria immitis</i> infection

that may contain numerous small vacuoles and few pink-to-azurophilic granules (lysosomes). Amoeboid or short hairlike processes (pseudopodia) may bulge from the cytoplasmic membrane.³⁹

Monocytes occur in low numbers in the blood of healthy dogs. Monocytosis is defined by numbers that exceed 1,400 monocytes/ μL of blood.³⁹ In general, monocytosis occurs with neutrophilia, but rarely it occurs as a single alteration in a leukogram (Table 48.6).^{36,39} Monocytosis is a frequent leukogram alteration that is associated with necrosis, suppuration, malignant neoplasia, pyogranulomatous lesions, internal hemorrhage, hemolysis, trauma,³⁶ and immune-mediated diseases. It is an inconsistent finding in the leukogram of dogs responding to corticosteroids. It may be observed in leukograms of dogs that have bacterial endocarditis or bacteremia. Monocytosis is seen in dogs rebounding from neutropenia such as in cyclic

hemopoiesis of gray-collie dogs.³⁹ Significant, persistent monocytosis, with counts >50,000/ μ L of blood, may be detected in monocytic or myelomonocytic leukemia. Abnormal monocyte morphology and numerous blast cells can be detected in the blood or bone marrow during these neoplastic diseases.

Causes and Mechanisms of Monocytopenia

Reference intervals for monocyte counts in the blood from healthy dogs are fairly broad. Therefore, it is difficult to document monocytopenia. Thus, this alteration is of little clinical significance. However, monocytopenia may be detected in some cases of acute pancytopenia due to several causes.³⁶

LEUKEMIA

The definition of leukemia is the neoplastic proliferation of hemopoietic cells originating in the bone marrow (see Section V). Leukemias (i.e. myeloproliferative disorders) are classified by cell type and differentiation, time course of disease process (acute or chronic), and presence or absence of neoplastic cells in circulation. Myeloproliferative disorders reported in the dog include granulocytic (myeloid) leukemia, myelomonocytic leukemia, monocytic leukemia, basophilic leukemia, mast cell leukemia, erythremic myelosis, polycythemia vera, megakaryocytic leukemia, and essential thrombocythemia. Lymphoproliferative diseases reported in the dog include lymphocytic leukemia and plasma cell leukemia. Diagnosis of these disorders is facilitated by examination of Romanowsky-stained blood films and bone marrow aspirates. Histologic examination of bone marrow core biopsies may be required in some cases. Use of immunohistochemistry greatly aids diagnosis in poorly differentiated cell types.^{31,36,39,40}

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Interpretation of Feline Leukocyte Responses

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Leukocyte Morphologic Artifacts	Neutropenia
Nonpathologic Leukocyte Responses	Increased use or destruction
Physiologic Leukocytosis	Deficient production
Stress Leukogram	Redistribution and sequestration
Pathologic Leukocyte Responses	Monocyte Responses
Inflammatory Leukogram	Monocytosis
Inflammatory Leukogram with a Left Shift	Monocytopenia
Neutrophil Responses	Lymphocyte Responses
Pathologic Changes in Neutrophil Morphology	Lymphocytosis
Toxic change	Lymphopenia
Pelger-Huët anomaly	Eosinophil Responses
Hypersegmented neutrophils	Eosinophilia
Mucopolysaccharidosis	Eosinopenia
Chédiak-Higashi syndrome	Basophil Responses
Cytoplasmic vacuolization	Basophilia
Neutrophil granulation anomaly	Basopenia
Intracellular infectious agents	Leukemia (Myeloproliferative and Lymphoproliferative Disorders)
Intracellular pigment	Myelodysplastic Syndrome
Neutrophilia	Aplastic Pancytopenia (Aplastic Anemia)

Acronyms and Abbreviations

CBC, complete blood count; CNP, circulating neutrophil pool; EDTA, ethylenediaminetetraacetic acid; FeLV, feline leukemia virus; FIV, feline immunodeficiency virus; MDS, myelodysplastic syndrome; MPD, myeloproliferative disease; nRBC, nucleated red blood cell; WBC, white blood cell.

The complete blood cell count (CBC) is a vital component of the minimum laboratory database used in the evaluation of feline illnesses. The CBC provides quantitative and qualitative information regarding the status of red blood cells (RBCs), platelets, and white blood cells (WBCs); it is also termed the leukogram. The leukogram includes a total WBC count with absolute and relative quantification of WBC types and comments on WBC morphology. Although some general rules regarding leukogram interpretation may be applied to all species, some unique characteristics must be considered and recognized during evaluation of the feline leukogram.

In normal cats, the total WBC population is composed primarily of mature neutrophils and lesser numbers of lymphocytes. Monocytes, eosinophils, and

basophils usually do not contribute significantly to the total WBC count. Chapter 105 offers a detailed review of normal feline WBC morphology.

LEUKOCYTE MORPHOLOGIC ARTIFACTS

Peripheral blood samples that are collected in EDTA anticoagulant and allowed to sit for several hours before a blood smear is made tend to develop artifactual leukocyte nuclear hypersegmentation, pyknosis, or cytoplasmic vacuolization (Fig. 49.1). Once these changes occur, the cells are difficult or impossible to identify. Artifactual cytoplasmic vacuolation also may be confused for toxic changes of neutrophils. When nucleated cells rupture or are stripped of their cytoplasm during

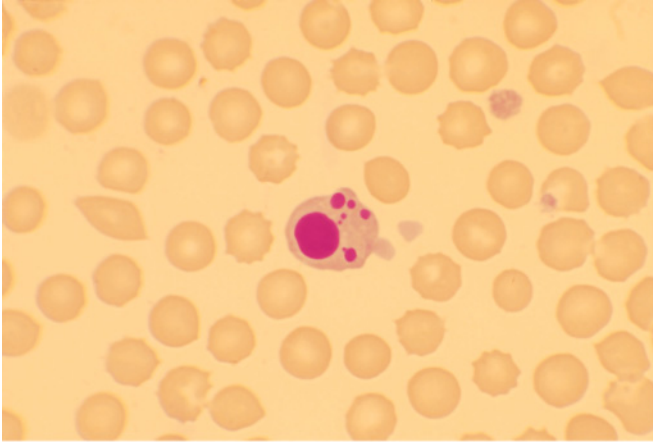


FIGURE 49.1 Improper sample handling has resulted in altered WBC morphology. A WBC shows pyknosis, karyorrhexis and karyolysis. Wright-stained blood smear. (Courtesy of Oklahoma State University, College of Veterinary Medicine teaching files.)

blood smear preparation, the nuclear chromatin spreads out and stains eosinophilic. These eosinophilic masses are referred to as basket cells. Attempts should not be made to identify basket cells. When more than 10% of WBCs are artifactually affected, the WBC differential count is invalid.

NONPATHOLOGIC LEUKOCYTE RESPONSES

Various physiologic and pharmacologic processes can cause changes in the total leukocyte count. Because neutrophils are the predominant blood leukocyte in the cat, alterations in the WBC count frequently parallel alterations in the absolute neutrophil count. Nonpathologic leukogram changes primarily result from shifts in neutrophil populations related to changes in neutrophil kinetics. Granulopoiesis is directed by cytokines primarily secreted from macrophages within the bone marrow. Maturation of neutrophils occurs in approximately 3.5–6 days.¹⁸ Three distinct pools of neutrophils exist. The first pool is the bone marrow pool of developing and mature neutrophils. Neutrophils are released from the bone marrow in an age-ordered fashion. Once in the systemic circulation, they are distributed between the marginated neutrophil pool and the circulating neutrophil pool (CNP). Marginated neutrophils adhere to endothelium of venules and capillaries primarily in the spleen, lungs, and splanchnic vessels, whereas neutrophils of the CNP remain free in the vasculature. Only circulating neutrophils are collected during venipuncture sampling. In the cat, the ratio of the marginated neutrophil pool to circulating neutrophil pool is approximately 3:1.⁹ Blood neutrophils remain in a dynamic equilibrium, and many processes can cause a redistribution of neutrophils between these pools. Shifts are dynamic and may occur rapidly, altering circulating neutrophil numbers as reflected by the total WBC and neutrophil counts. Two distinct patterns of nonpathologic leukograms have been identified.

Physiologic Leukocytosis

In physiologic leukocytosis, a mild to moderate leukocytosis characterizes the leukogram. Mild, mature neutrophilia and lymphocytosis exist. The lymphocytosis is frequently of greater magnitude than the neutrophilia.^{9,18} Lymphocytosis is probably caused by redistribution of lymphocytes between the blood, lymphatics, and lymphoid organs. The neutrophilia reflects the redistribution of neutrophils from the marginated to the circulating pool due to the effects of epinephrine. This leukogram pattern is generally seen in young, healthy cats that become excited or frightened by environmental stresses (e.g. fear, excitement, restraint, venipuncture). Leukogram changes are immediate but transient, lasting approximately 20–30 minutes. The short duration of the physiologic lymphocytosis helps to distinguish this physiologic state from other pathologic processes that cause a persistent lymphocytosis (e.g. lymphoma and lymphocytic leukemia). Young cats typically have a higher number of circulating lymphocytes than older cats, and a physiologic lymphocytosis rarely exceeds 20,000 lymphocytes/ μL of blood.⁹

Stress (Glucocorticoid-Induced) Leukogram

Exogenous glucocorticoids or elevations in endogenous glucocorticoids tend to cause a mild to moderate leukocytosis characterized by a mature neutrophilia, lymphopenia, and eosinopenia. Monocytosis generally is not observed in cats, and an eosinopenia is difficult to document in any species. Glucocorticoids induce neutrophilia primarily by enhancing the release of mature neutrophils from the bone marrow. Secondarily, glucocorticoids promote the shift of marginated neutrophils into the circulating neutrophil pool, decreasing the egress of circulating neutrophils into tissue. The normal blood transit time of circulating neutrophils is approximately 10–12 hours, but this blood transit time is prolonged by glucocorticoid administration. Finally, chronic glucocorticoid excess stimulates granulopoiesis. The lymphopenia is the result of redistribution of circulating lymphocytes; lysis of immature or uncommitted lymphocytes may occur with high dosages of corticosteroids given over prolonged periods. Glucocorticoids also exert a neutralizing effect on histamine, a major chemoattractant for eosinophils.¹³ The corticosteroid response is initially seen at approximately 4 hours and peaks 6–8 hours post-stress or after administration of corticosteroids. Neutrophil values return to reference intervals within 24 hours after a single 5 mg dose of prednisolone and within 48–96 hours after cessation of prolonged corticosteroid therapy.¹⁸

PATHOLOGIC LEUKOCYTE RESPONSES

Because neutrophils are the predominant leukocyte in the cat, alterations in the total WBC count frequently parallel alterations in neutrophil numbers. Many factors, including physiologic, pharmacologic, and

pathologic processes can cause changes in the absolute neutrophil count in blood.

Inflammatory Leukogram

In a normal animal, homeostasis is maintained between production and egress of neutrophils into tissues. Bone marrow production, margination, and tissue demand for neutrophils all can be affected by various disease processes. With an increased tissue demand for neutrophils, the bone marrow responds by increasing the release of mature neutrophils into circulation. In the cat, a significant, mature neutrophilic leukocytosis indicates inflammation. With established inflammation, a sustained neutrophilia indicates that bone marrow release of mature neutrophils exceeds neutrophil emigration into tissues. Bone marrow evaluation usually reveals granulocytic hyperplasia. These findings reflect the bone marrow's capacity to produce enough mature neutrophils to combat the inciting agent and also suggests that the inflammatory process has been present for at least several days. The demand for neutrophils depends on the chronicity and severity of the disease process.

Inflammatory Leukogram With a Left Shift

When the demand for neutrophils exceeds bone marrow reserve capacity, the storage pool of mature neutrophils becomes exhausted. Immature neutrophils are then released into the circulation. An increase in the number of band cells and occasionally earlier precursor cells into the blood is referred to as a left shift. A clinically significant left shift is the hallmark of an inflammatory leukogram. In the cat, a left shift is considered clinically significant when immature neutrophils exceed 500–1000 bands/ μL with a normal or elevated total WBC count.²² A left shift may be categorized as either regenerative or degenerative, which may be useful clinically as a prognostic indicator of disease severity. Additionally, the severity of the left shift can be assessed by evaluating the numbers of immature granulocytes and degree of immaturity of the cells, as an increase in both parameters generally parallels the severity of the condition. A regenerative left shift is defined as a neutrophilic leukocytosis in which the number of immature neutrophils does not exceed the number of segmented neutrophils. A regenerative left shift indicates that the bone marrow is meeting the body's need for neutrophils. In contrast, a degenerative left shift is present when the number of immature neutrophils exceeds the number of segmented neutrophils.¹³ The number of segmented neutrophils is typically within the reference interval or decreased. A degenerative left shift indicates that the bone marrow cannot meet the tissue demand for neutrophils, indicating a severe disease process and suggests a guarded to poor prognosis. Degenerative left shifts can occur in conditions such as septicemia, endotoxemia, and severe inflammation of large-surface areas (e.g. peritonitis, pleuritis, pneumonia, gastroenteritis,

placentitis). Frequently, a left shift is limited to band neutrophils, but with severe disease, metamyelocytes, myelocytes, and promyelocytes (progranulocytes) can appear in the blood. These immature stages are more likely to be associated with a degenerative left shift than with a regenerative left shift. Giant neutrophils or metamyelocytes are more frequent in the cat than in other species and can be seen as cats recover from severe neutropenia. Toxic neutrophils are frequently seen with inflammatory responses and are discussed later. Occasionally, an inflammatory process may induce an extreme neutrophilia (>50,000 cells/ μL) with an associated left shift that may include band, metamyelocyte, myelocyte, and promyelocyte stages. This is termed a leukemoid response and can be difficult to distinguish from granulocytic or myelomonocytic leukemia (see Section V).

NEUTROPHIL RESPONSES

Pathologic Changes in Neutrophil Morphology

Toxic Change

Toxic change occurs in neutrophils during maturation in the bone marrow and primarily affects the myelocytic and metamyelocytic stages of development. Toxic change is a maturational defect caused by toxic substances generated by strong inflammatory conditions including severe bacterial infections (pyothorax, pyoabdomen, sepsis, pneumonia), septicemia, acute inflammatory conditions, and extensive burns. Toxic changes can be seen in both mature and immature neutrophils and generally are associated with a left shift. Toxicity can be observed before quantitative changes in the leukogram, thus serving as a harbinger of disease, and may be the only hematological change evident to indicate an infectious/inflammatory condition.²³ Toxic change can be present with a neutrophilia or a neutropenia and with or without a left shift. Toxicity is most frequently secondary to systemic rather than localized infectious/inflammatory conditions.^{26,29}

Although both nuclear and cytoplasmic toxic changes occur, the cytoplasmic changes are more reliable and occur more frequently. Although all cell types are exposed to the same insult, toxic change is evaluated only in neutrophils. The type of toxic change is indicated and semiquantitated as either 1+ to 4+, or as slight, mild, moderate, or severe. Cytoplasmic toxic changes include diffuse cytoplasmic basophilia, cytoplasmic vacuolation, Döhle bodies, and toxic granulation, with the latter change infrequently observed. Diffuse cytoplasmic basophilia occurs when polyribosomes are retained in the cytoplasm. Cytoplasmic vacuolation generally occurs in association with diffuse basophilia (Fig. 49.2). The mechanism of cytoplasmic vacuolization is thought to stem from damage to the cell membrane and subsequent loss of integrity.^{26,29} Döhle bodies are intracytoplasmic clumps of endoplasmic reticulum that stain a light blue with most Romanowsky-

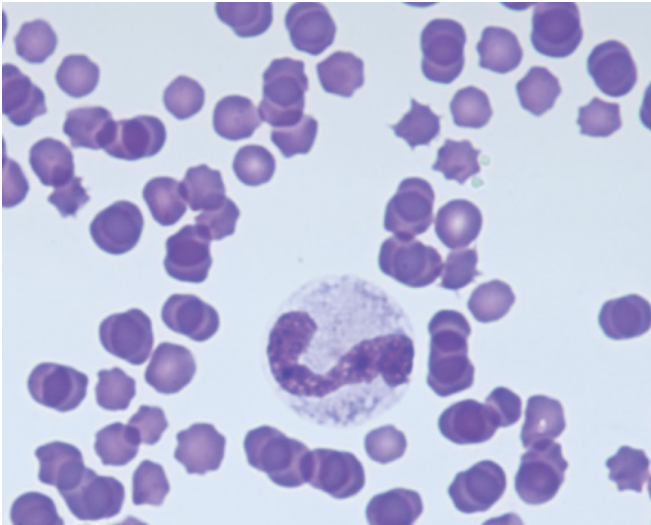


FIGURE 49.2 Feline neutrophil showing toxic change denoted by increased cytoplasmic basophilia, Döhle bodies, and foamy cytoplasm. Wright-stained blood smear. (Courtesy of IDEXX Reference Laboratories teaching files.)

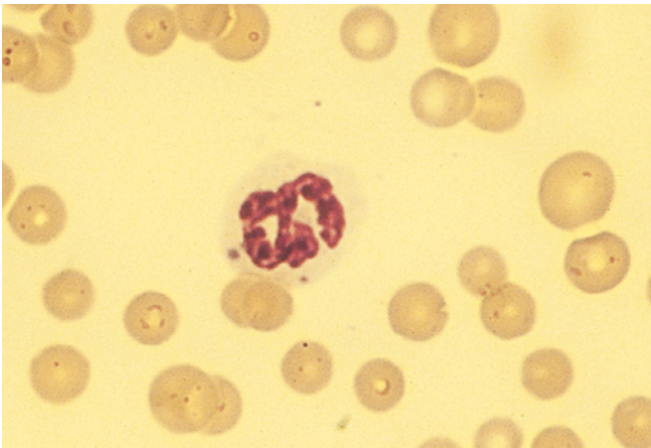


FIGURE 49.3 A feline neutrophil contains Döhle bodies. Wright-stained blood smear. (Courtesy of Oklahoma State University, College of Veterinary Medicine teaching files.)

type stains (Fig 49.3). Döhle bodies occur frequently in cat neutrophils. Regardless of the number of neutrophils containing Döhle bodies, their presence should never be interpreted as indicating anything more than a mild toxic change. Toxic granulation occurs when primary granules within neutrophils retain sufficient amounts of mucopolysaccharide so that these granules stain reddish-purple. Nuclear toxic changes can include: vacuolization, hyposegmentation, ring formation, karyorrhexis, karyolysis and giant neutrophils.^{26,29}

Pelger-Huët Anomaly

Pelger-Huët anomaly is an inherited or acquired (pseudo Pelger-Huët) defect in granulocyte nuclear segmentation (see Chapter 42).^{19,20,31} In Pelger-Huët

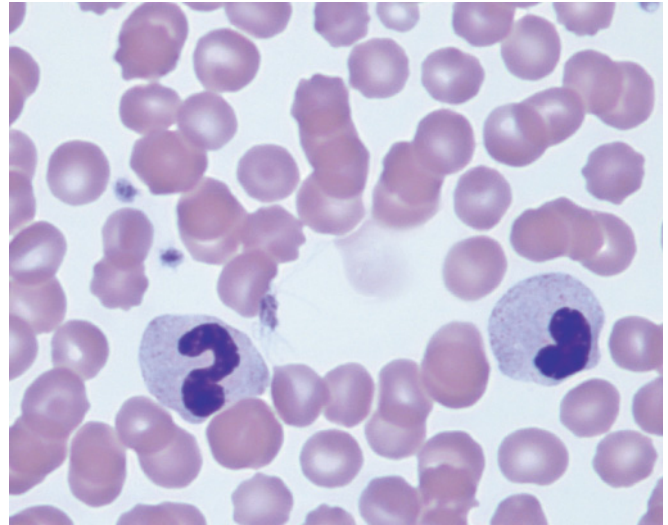


FIGURE 49.4 Pelger-Huët anomaly. Two neutrophils show mature chromatin patterns and lack of segmentation. Wright-stained blood smear. (Courtesy of IDEXX Reference Laboratories teaching files.)

anomaly, hyposegmented nuclei characteristically have a mature chromatin pattern, in contrast to nuclear hyposegmentation of infection where band cells have finely granular chromatin (Fig. 49.4). The lack of toxic change in the face of a significant degenerative left shift is also a hint to consider Pelger-Huët anomaly, and to carefully assess the nuclear chromatin pattern of the granulocytes. Neutrophil function is normal in affected cats, and cats with this anomaly are not predisposed to infection.

Hypersegmented Neutrophils

Hypersegmented neutrophils contain ≥ 5 distinct nuclear lobes. Hypersegmentation can occur as an *in vivo* aging change caused by prolonged blood transit time. More frequently, hypersegmentation, as well as pyknosis, represents an *in vitro* artifactual aging change that is seen in smears of EDTA-anticoagulated blood that has been allowed to sit for several hours.

Mucopolysaccharidosis

Mucopolysaccharidosis is a metabolic storage disease caused by inborn errors of mucopolysaccharide (glycosaminoglycan) metabolism.^{6,7,10,11} Circulating neutrophils contain few to many coarse, reddish-purple, intracytoplasmic granules on Romanowsky-stained blood smears (Fig. 49.5). Basophilic granules frequently are enlarged and metachromatic.

Chédiak-Higashi Syndrome

This syndrome occurs in Persian cats (see Chapter 42).^{15,16} Circulating neutrophils and lymphocytes contain

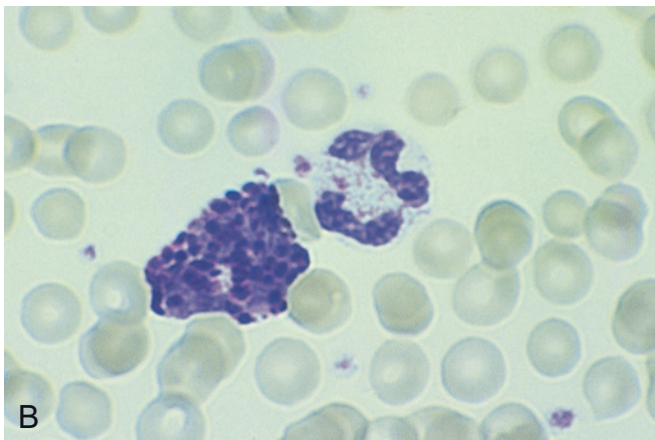
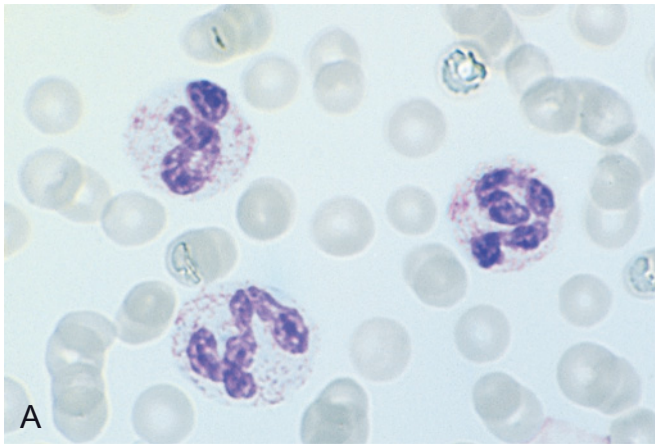


FIGURE 49.5 Blood smear from a cat with mucopolysaccharidosis type VI. (A) Three neutrophils contain granular, purple, intracytoplasmic inclusions. (B) A neutrophil with intracytoplasmic inclusions and a basophil with dark blue-black granules. Wright stain. (Courtesy of Oklahoma State University, College of Veterinary Medicine teaching files.)

single to multiple, pink to reddish, intracytoplasmic granules (Fig. 49.6). Eosinophilic granules also may be enlarged.

Cytoplasmic Vacuolization

Cytoplasmic vacuolization of neutrophils can be observed with drug toxicities including high doses of chloramphenicol or phenylbutazone. Also, cholesteryl ester storage disease is associated with subtle cytoplasmic vacuolation of neutrophils, lymphocytes, and monocytes on fresh blood smears.²⁸ Neutrophil cytoplasmic granulation is also seen after storage of blood in EDTA.

Neutrophil Granulation Anomaly

A neutrophil granulation anomaly has been identified in Birman cats (see Chapter 42). The anomaly is characterized by the presence of azurophilic cytoplasmic granules that resemble toxic granulation.¹¹ The

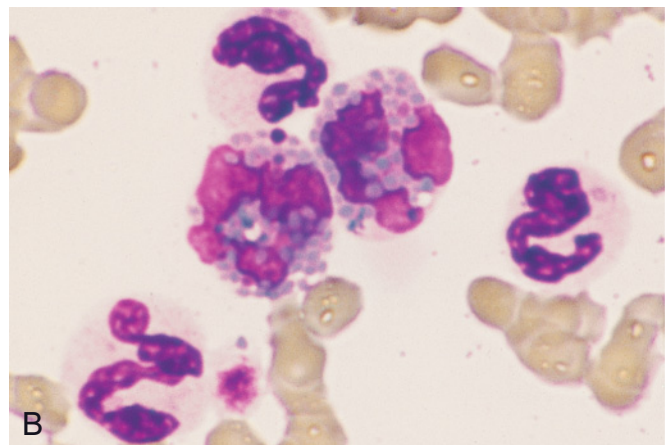
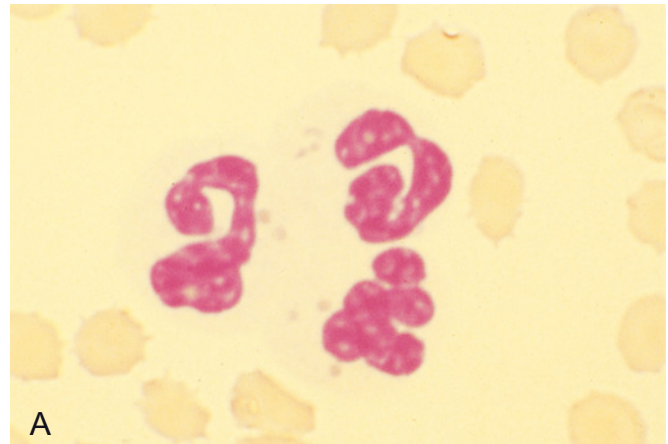


FIGURE 49.6 Blood smear from a cat with Chédiak-Higashi syndrome. (A) The neutrophils have large, pink cytoplasmic lysosomes. (B) The basophils have large, round, lavender granules. Wright stain. (Courtesy of Oklahoma State University, College of Veterinary Medicine teaching files.)

anomaly is inherited as an autosomal recessive trait and is innocuous.

Intracellular Infectious Agents

Histoplasma capsulatum yeast can rarely be seen in blood and buffy coat leukocytes in cats with disseminated histoplasmosis (see Chapter 19). These organisms are identified by their small size and round shape. They are 2–4 μm in diameter with a darkly staining, eccentrically located nucleus and a thin clear cell wall that resembles a halo (Fig. 49.7). The presence of intracellular bacteria is uncommon, and could be consistent with contamination of the specimen or with sepsis. Correlation with clinical signs, history, laboratory data, and results of blood culture are necessary to distinguish between these differentials. One or more morulae of *Ehrlichia* spp. are rarely identified in the cytoplasm of circulating neutrophils or monocytes in cats (see Chapter 31). Morulae are found in cytoplasmic vacuoles, are round with an approximate diameter of

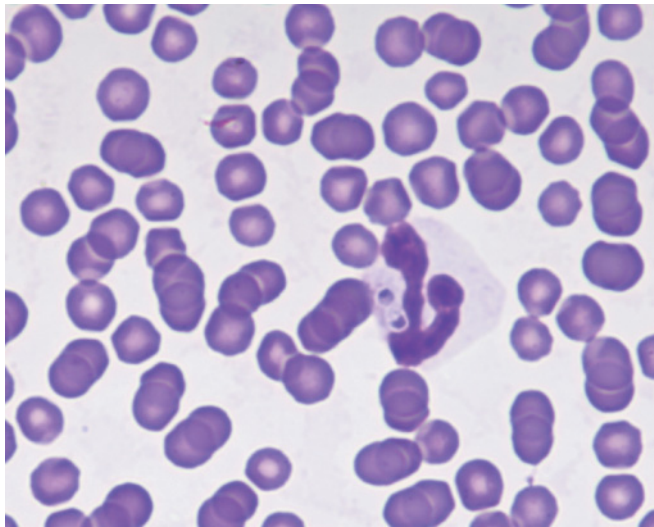


FIGURE 49.7 Feline neutrophil with two intracellular yeast forms of *Histoplasma capsulatum*. Wright-stained blood smear. (Courtesy of IDEXX Reference Laboratories teaching files.)

1.5–4 μ m, and are composed of many small intracellular organisms that stain basophilic with Wright stains.

Intracellular Pigment

Rarely in hemolytic anemia, circulating sideroleukocytes can be identified. Sideroleukocytes are neutrophils and occasionally monocytes with intracellular hemosiderin, staining either brown-yellow, or blue-green on Romanowsky-stained blood films. The presence of intracellular hemosiderin can be confirmed with iron stains.

Neutrophilia

As previously stated, blood neutrophil counts may be increased by physiologic leukocytosis, corticosteroid-induced leukocytosis or pathologic mechanisms. Inflammation is the most common cause of a pathologic neutrophilia. Inflammation may stem from infectious or noninfectious origins. Infectious disease processes include bacterial, viral, fungal, and parasitic infections that can be localized or generalized (systemic).¹⁸ The magnitude of the neutrophilia and the presence or absence of a left shift depends on the severity and duration of disease, as well as the competence of the bone marrow to produce and release neutrophils. With prolonged duration, localized inflammatory processes may result in very high neutrophil counts. These cases are frequently associated with a left shift and toxic change of neutrophils. Occasionally, the inflammatory nidus is obscure. In such cases, exudative loss of neutrophils into large-surface areas should be considered. Such sites of loss include the skin, gastrointestinal tract, genitourinary tract, respiratory tract, and joints. Surgical removal of the inciting cause (e.g. abscess) often results in a transient increase in neutrophil numbers due to

granulocytic hyperplasia in the bone marrow. Medical treatment involving the administration of glucocorticoids may induce a transient glucocorticoid (stress) leukogram. Generalized or systemic infectious processes also can cause a neutrophilia, but the magnitude of the neutrophilia is generally less severe than localized infectious processes. Some specific examples include feline viral rhinotracheitis and feline infectious peritonitis. These diseases infrequently present with neutropenia.

Various noninfectious causes of tissue necrosis can induce mild to moderate neutrophilia. Any process that leads to tissue necrosis can result in an inflammatory response. As expected, the magnitude of the neutrophilia and the presence of a left shift depend on disease severity and chronicity. A few examples include soft-tissue trauma, immune-mediated diseases (e.g. hemolytic anemia), hemorrhage, and nonleukemic neoplasia.

Neutropenia

Neutropenia (<2500 neutrophils/ μ L) can result from several causes and various mechanisms.⁹ General mechanisms of neutropenia include increased tissue use or destruction in which neutrophil egress or loss exceeds bone marrow production, decreased bone marrow production or release of neutrophils, and redistribution or sequestration of neutrophils. A single cause (e.g. endotoxemia) may act by several different mechanisms to produce neutropenia.

Increased Use or Destruction

When tissue demand for neutrophils exceeds replacement by the bone marrow, neutropenia occurs. Acute inflammatory processes frequently cause massive emigration of neutrophils into tissue. Acute or peracute bacterial infection of highly vascular organs is the most frequent cause of increased tissue use of neutrophils in cats. With time, inflammatory responses secondary to bacterial infections generally progress to a neutrophilic leukocytosis, assuming there is adequate bone marrow response. Endotoxemia, associated with bacterial infections, induces a neutropenia by several mechanisms. Neutrophil circulation time is decreased and egress into tissues is enhanced. Endotoxemia also activates complement and production of complement fragment C5a induces intravascular neutrophil aggregation (leukoagglutination) and subsequent neutrophil destruction. In addition, excessively high body temperature (e.g. heatstroke) may result in the destruction of neutrophils and neutrophil precursors in the bone marrow.

Deficient Production

Decreased bone marrow production of neutrophils can occur secondary to variety of causes. Basic mechanisms include hemopoietic stem cell death, seen with certain infectious agents (e.g. FIV, FeLV and feline parvovirus; see Chapter 19) and some drugs (e.g. chloramphenicol,

cytotoxic drugs; see Chapter 16), as well as myelophthiic diseases (e.g. neoplasia and myelofibrosis) with subsequent loss of hemopoietic space. Although bone marrow suppression usually involves all hemopoietic elements (pancytopenia), the earliest change in the blood is a neutropenia.

Approximately 50% of cats that have FeLV-related illness present with a neutropenia and are leukopenic (see Chapter 60).^{9,18,22} Three FeLV-associated leukogram patterns have been described. A mild neutropenia with normal marrow cellularity is the most frequent form. A moderate to severe neutropenia, with concurrent marrow granulocytic hypoplasia is referred to as a panleukopenia-like syndrome. Presentation with a severe, persistent neutropenia and concurrent bone marrow granulocytic hyperplasia with dysplastic changes has been referred to as myelodysplasia (see Chapter 66). Mechanisms of neutropenia include virus-induced stem cell destruction, and retarded maturation, survival, and release of neutrophils from the bone marrow. Cyclic hemopoiesis, characterized by a neutropenia with cycle lengths ranging from 8 to 16 days, has been documented in FeLV-positive cats.²⁷ Neutropenia has been seen in cats infected with FIV, an immunosuppressive retrovirus (see Chapter 55).

Administration of drugs including chemotherapeutic drugs and chloramphenicol, can induce neutropenia in cats by suppression of bone marrow granulopoiesis (see Chapter 16). Toxic changes, notably Döhle bodies and cytoplasmic vacuolation, can be present in blood neutrophils. Chloramphenicol toxicity is seen with high dosages or with chronic administration of lower dosages.³⁰

Neutropenia can be seen with many myelophthiic diseases, including leukemias, certain granulomatous inflammatory conditions (i.e. disseminated histoplasmosis), metastatic neoplasms, myelofibrosis, and osteosclerosis (i.e. bone deposition, see Chapter 18). Myelophthiic diseases frequently affect other hemopoietic cell lines, resulting in a panleukopenia (nonregenerative anemia, thrombocytopenia, and leukopenia). Examination of a bone marrow aspirate and core biopsy may provide a definitive diagnosis when myelophthiic disease is suspected.

Ehrlichiosis in cats is associated with neutropenia, usually with concurrent anemia and thrombocytopenia (see Chapter 19). Intracellular *Ehrlichia morulae* are uncommonly identified in neutrophils and monocytes. Bone marrow findings are consistent with hypoplasia with evidence of dysplasia.²

Redistribution and Sequestration

Pathologic processes may cause sequestration or shifts in neutrophil populations. Early endotoxemia enhances margination of circulating neutrophils, and a rapid, marked neutropenia may ensue after initial endotoxin exposure. This effect is transient, and frequently is followed by a neutrophilia within 1–3 hours.¹⁸ Sequestration of neutrophils may be seen post-transfusion or with processes such as hypersplenism.

MONOCYTE RESPONSES

The bone marrow does not contain a storage pool of monocytes. Thus, the bone marrow transit time is short, approximating 1–2.5 days. After monocytes enter the blood, they exit the vasculature randomly. The blood half-life of monocytes is 8.4 hours.

Monocytosis

An absolute monocyte count >850 cells/ μ L is considered a monocytosis.^{9,18} Monocytosis is a nonspecific finding that has been reported to occur in approximately 11% of the leukograms of hospitalized cats.¹⁸ Monocytosis is not a characteristic feature of the stress leukogram in cats as it is in dogs. Monocytosis occurs in many conditions, including both acute and chronic inflammation, tissue destruction, and neutrophilia. Some of the reported causes of a monocytosis include trauma-related injuries, suppuration, necrosis, pyogranulomatous inflammation, hemolysis, hemorrhage, malignancy, and immune-mediated disorders.

Monocytopenia

Persistent monocytopenia is clinically unimportant in cats and is seldom documented, because the low end of the reference interval is zero cells/ μ L of blood. With severe leukopenia, the clinical focus is on neutropenia and prevention of sepsis; little attention is given to coexisting monocytopenia.

LYMPHOCYTE RESPONSES

Lymphocytes are the second most frequent leukocyte in the blood of healthy cats. Most blood lymphocytes are small-sized, long-lived, T cells that are capable of recirculation. Antigenically-stimulated lymphocytes (e.g. reactive lymphocytes) are larger and have more abundant, dark-blue cytoplasm. Rarely, immune-stimulated lymphocytes may exhibit plasmacytoid differentiation. Such cells have a round, eccentric nucleus with coarse nuclear chromatin; abundant dark-blue cytoplasm; and a pale-staining Golgi zone located between the nucleus and the largest volume of cytoplasm. Granular lymphocytes, that are natural killer cells and in the null cell group, may be observed. These cells are identified by distinctive azurophilic granules, usually in the vicinity of the nuclear indentation. In contrast, lymphoblasts (immature lymphocytes) are large lymphocytes (equal to or larger than neutrophils) with finely stippled nuclear chromatin and multiple, prominent nucleoli. Lymphocyte vacuolation has been associated with cholesteryl ester storage disease of Siamese cats and mannosidosis of Persian cats.^{21,28}

Lymphocytes are evaluated by their morphology and absolute number. Blood lymphocyte numbers are influenced by physiologic states, disease, and drug administration, causing changes in lymphocyte

production, distribution, margination, recirculation, sequestration, destruction, or loss.

Lymphocytosis

Lymphocytosis (>7000 lymphocytes/ μL) can be secondary to epinephrine-mediated physiologic leukocytosis, hypoadrenocorticism, chronic antigenic stimulation (e.g. FeLV), hyperthyroidism, or lymphoid neoplasia. Young animals also have higher absolute lymphocyte counts compared to middle-aged or old cats.⁹ Young, healthy cats (especially those <1 year of age) are especially prone to lymphocytosis secondary to excitement or fear (physiologic leukocytosis). With physiologic leukocytosis, the absolute lymphocyte count is usually less than 20,000 lymphocytes/ μL but has been reported to exceed 36,000 lymphocytes/ μL .¹⁸ Physiologic lymphocytosis is caused by epinephrine and is short lived. If another blood sample can be collected from the cat after it has been allowed to calm down, the absolute lymphocyte count should be within the reference interval, allowing differentiation from a pathologic cause of lymphocytosis. Lymphocytosis also is present in 20% of cats that have glucocorticoid deficiency (hypoadrenocorticism).¹⁸

Chronic antigenic stimulation causes lymphocyte proliferation and may result in lymphocytosis. An example of chronic antigenic stimulation is the lymphocytosis seen with FeLV-associated peripheral lymph node hyperplasia. Lymphocytosis also may be seen a few days post-vaccination. Reactive lymphocytes (immunocytes) are a more frequent finding with antigenic stimulation than is lymphocytosis. Reactive lymphocytes, when present, must be differentiated from atypical (immature) lymphocytes that would suggest lymphoid neoplasia.

Lymphopenia

Lymphopenia is recognized by an absolute lymphocyte count of less than 1,500 lymphocytes/ μL of blood in adult cats or less than 2,500 lymphocytes/ μL of blood in young cats.^{9,18} The most frequent cause of lymphopenia is corticosteroid-induced redistribution of lymphocytes. Some other causes include viral infections (e.g. panleukopenia, FeLV, and FIV), septicemia or endotoxemia, lymphocyte-rich thoracic effusions (chylothorax from thoracic duct rupture, cardiovascular disease, nonexfoliating neoplasia, malignant lymphoma, or thymoma), and gastrointestinal disease (e.g. ulcerative enteritis, granulomatous enteritis, malignant lymphoma, and other neoplasias).

EOSINOPHIL RESPONSES

Like neutrophils, the bone marrow has a storage pool of eosinophils. Eosinophils have many functions, including the destruction of parasites, the modulation of hypersensitivity reactions, and a proinflammatory function.

Eosinophilia

The absolute number of eosinophils that constitutes an eosinophilia varies by geographic areas, but it generally represents absolute eosinophil counts in excess of 750–1,500 eosinophils/ μL . Some causes of eosinophilia include hypersensitivity or inflammatory lesions (e.g. ulcerative gastroenteritis, food allergy, flea allergy dermatitis, atopy, eosinophilic keratitis, feline eosinophilic granuloma complex, bronchial asthma), parasites (endoparasites and ectoparasites), idiopathic hypereosinophilic syndrome, FeLV-associated eosinophilia, tumor-associated eosinophilia (mast cell neoplasia, carcinomas, malignant lymphoma, myeloproliferative disease)^{1,4,17,24} and miscellaneous conditions (e.g. hyperthyroidism and hypoadrenocorticism).^{3,4,9,18} Eosinophilic leukemia and idiopathic hypereosinophilic syndrome result in a marked eosinophilia (>50,000 eosinophils/ μL).⁵

Eosinopenia

Eosinopenia is difficult to recognize clinically because the lower end of the reference interval for feline eosinophil counts is 0 cells/ μL . Eosinopenia usually is associated with exogenous administration or stress-related endogenous release of glucocorticoids. Acute infections are associated with eosinopenia, but this is likely glucocorticoid-induced.

BASOPHIL RESPONSES

Basophils are the least numerous of the blood leukocytes in healthy cats, accounting for less than 2% of the leukocyte differential count.¹⁸

Basophilia

A basophilia is recognized by an increased absolute basophil count (>200 basophils/ μL) and generally occurs with a concomitant eosinophilia. Some causes of basophilia in cats include allergic respiratory conditions, heartworm disease, eosinophilic granuloma complex, basophilic leukemia, myeloid leukemia, mast cell neoplasia and polycythemia vera.^{3,9,18}

Basopenia

Basophils are rarely observed in blood smears of cats; basopenia is not a recognized clinical problem.

LEUKEMIAS (MYELOPROLIFERATIVE AND LYMPHOPROLIFERATIVE DISORDERS)

In depth discussion, including pathogenesis, classification, general and detailed features of acute and chronic myeloid and lymphoid leukemias can be found in Section V. Leukemias and myeloproliferative disorders (MPDs) originate in the bone marrow. Most of the MPDs and leukemias in cats are FeLV-associated and

some also have been associated with FIV infection. Myeloproliferative disorders include neoplastic proliferation of granulocytes, monocytes, erythrocytes, and megakaryocytes. The acute MPDs include acute undifferentiated leukemia and the acute myeloid leukemias (French-American-British classification M1–M7).⁸ Chronic MPD categories include chronic myeloid leukemias, polycythemia vera, idiopathic myelofibrosis, myeloid metaplasia, and essential thrombocythemia.⁸

Leukemias are encountered less frequently than lymphoma in the cat.⁸ Cats that have leukemia generally have vague and variable clinical signs, including anorexia, loss of body mass, and lethargy. Clinical findings often include organomegaly, anemia, fever, emaciation, and petechiae.¹⁸ Leukemia usually is associated with a high total WBC count, but it occasionally may present with a normal WBC count or leukopenia. In chronic leukemias composed of well-differentiated cells, the type of leukemia often can be identified by examination of Wright-stained blood and bone marrow smears. However, in acute leukemias, blast cells can appear similar morphologically.¹⁴ Therefore, immunophenotypic assessment may be needed to identify the neoplastic cell line.¹⁴

Granulocytic, myelomonocytic, and monocytic leukemias all occur in cats. Granulocytic leukemia must be differentiated from a leukemoid response or extreme neutrophilic leukocytosis secondary to an inflammatory focus (e.g. infection and tumor with a necrotic center).

Eosinophilic leukemia is rare and difficult to differentiate from idiopathic hypereosinophilic syndrome. Basophilic leukemia also is rare and should not be confused with mast cell neoplasia. In cats, mast cell neoplasia usually is associated with splenic enlargement or gastrointestinal involvement and with circulating mast cells.

Lymphoproliferative disease includes acute lymphoblastic leukemia and chronic lymphocytic leukemia. The latter is characterized by many small, well-differentiated lymphocytes, and must be differentiated from a physiologic lymphocytosis and from a reactive lymphocytosis secondary to antigenic stimulation.

MYELODYSPLASTIC SYNDROMES

Hallmarks of myelodysplastic syndromes (MDSs) are: ineffective hematopoiesis leading to peripheral cytopenias and evidence of dysplasia in the peripheral blood or bone marrow (Fig. 49.8; see Chapter 66).³² Some cases of MDS in the cat occur secondary to FeLV infection, resulting in retroviral induced mutations in bone marrow stem cells. MDS in the cat is most frequently associated with a moderate to severe anemia, but bicytopenia and pancytopenia are common.¹² Dysplastic changes observed in peripheral blood neutrophils can include: giant neutrophils, ring-shaped nucleated neutrophils, and hypersegmented neutrophils.^{12,25} Transition into acute myeloid leukemia is frequent in some types of MDS.

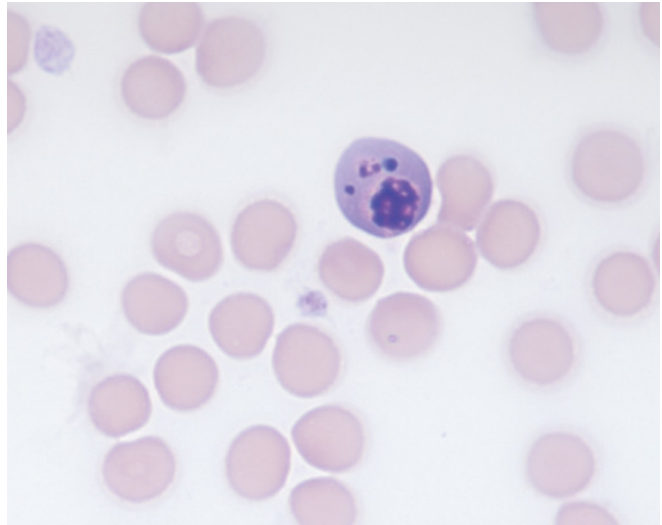


FIGURE 49.8 Blood smear of a dysplastic nucleated RBC. Wright stain. (Courtesy of IDEXX Reference Laboratories teaching files.)

APLASTIC PANCYTOPENIA (APLASTIC ANEMIA)

Similar to MDS, aplastic anemia results in peripheral cytopenias; however, the marrow is severely hypocellular or acellular and the hematopoietic space is replaced by adipose tissue (see Chapter 39). Etiologies of aplastic pancytopenia in the cat include: infectious agents (*Ehrlichia* sp., parvovirus, FeLV, FIV), drugs (chemotherapeutics, sulfadiazine, griseofulvin, albendazole) and idiopathic causes.³²

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Determination and Interpretation of the Avian Leukogram

KENNETH S. LATIMER and DOROTHEE BIENZLE

Leukocyte Count	Deficient Heterophil Production
Stained Blood Smear	Heterophil Shifts from the Circulating to Marginal Pool
Leukocyte Differential Count	Severe Tissue Demands for Heterophils
Changes in Leukocyte Morphology	Lymphocyte Responses
Toxic Changes	Lymphocytosis
Infectious Disease	Lymphopenia
Reference Intervals	Monocyte Responses
Hematopoiesis with Emphasis on Leukocyte Production	Monocytosis
Leukocyte Function and Response in Health and Disease	Monocytopenia
Heterophil or Lymphocyte Predominance in the Blood in States of Relative Health	Basophil Responses
Leukocytosis and Leukopenia	Basophilia
Leukocytosis	Basopenia
Leukopenia	Eosinophil Responses
Heterophil Responses	Eosinophilia
Physiologic Heterophilia	Eosinopenia
Corticosteroid-induced Heterophilia	Remarks on Prognosis and Hematologic Trends
Inflammation- or Infection-induced Heterophilia	Heterophil:Lymphocyte Ratios
Heteropenia	Unfavorable Prognostic Features
	Favorable Hematologic Trends
	New Techniques for Assessment of Avian Leukocytes

Acronyms and Abbreviations

ACTH, adrenocorticotrophic hormone; CBC, complete blood count; EDTA, ethylenediamine tetraacetic acid; H:L, heterophil:lymphocyte ratio; WBC, white blood cell.

Mammalian hematology has advanced rapidly over the past three decades owing to the introduction of semi-automated or automated technology (that provides a total leukocyte count and complete or 2- or 3-part differential leukocyte count). Published literature exists detailing clinical hematologic responses to disease in mammalian species. In contrast, avian hematology is still in its infancy. Descriptive accounts of avian blood-cell morphology^{9,39,42,44} and techniques of performing avian white blood cell (WBC) counts^{10,21,57,77} have been published. However, the progress of avian hematology has been inhibited by lack of automated technology, lack of

appropriate reference intervals for most major avian species, and lack of carefully controlled experimental studies to interpret avian WBC responses. Presented in this chapter is a brief overview of avian WBCs with respect to quantitation of WBC data, establishment of reference intervals, and interpretation of WBC responses in health and disease. Much of the research to date has been done in domestic poultry, which provides a model for avian WBC development and function.⁴³ Although generalizations can be drawn concerning avian leukocyte production, morphology, function, and response in health and disease, differences are sometimes apparent among and between species of birds.

LEUKOCYTE COUNT

In avian hematology, only the red blood cell (RBC) count can be determined routinely by automated methods (electronic particle counter).²¹ Therefore, three manual approaches are used to determine or estimate the WBC count. These include (1) the direct WBC count with a hemacytometer and Natt and Herrick's solution, (2) the indirect WBC count using a hemacytometer and eosinophil Unopette® in conjunction with the stained blood smear, and (3) the estimated WBC count using the stained blood smear. Specific technical procedures for these counting methods can be found in the literature.^{9,22,33,36,77}

The avian WBC count can be determined directly with a Neubauer-ruled hemacytometer, pipettes, and methyl violet 2B diluent.⁵⁷ The advantage of this technique is that all WBCs are visualized and enumerated. The disadvantages are that this WBC counting technique is labor intensive, care must be taken to differentiate thrombocytes from small lymphocytes, and WBCs may distort if analysis of the diluted specimen is delayed.⁴³ This method works well for an in-house laboratory but may prove difficult with samples that are mailed to a diagnostic laboratory. In the latter instance, prolonged exposure to ethylenediaminetetraacetic acid (EDTA) anticoagulant can cause erythrolysis and viscosity changes of hematologic specimens from crowned cranes, crows, jays, brush turkeys, hornbills, magpies, and some ratites.^{21,64} In addition, WBC morphology can be altered upon prolonged exposure to EDTA. Specimens collected in heparin eventually clot, and heparin also interferes with Romanowsky staining of blood smears.

The WBC count can be quantitated indirectly with the eosinophil Unopette #5877 system. In this technique, heterophils and eosinophils are selectively stained by phloxine dye and counted in a hemacytometer.^{10,65} Based on the WBC differential cell count of a stained blood smear, the combined heterophil and eosinophil hemacytometer count is mathematically corrected to account for basophils and mononuclear cells (i.e. lymphocytes and monocytes) that were not counted. Thus, the total WBC count per microliter of blood has been determined indirectly. Advantages of this system are that heterophils and eosinophils are directly identified and counted in a hemacytometer. There also is no confusion between lymphocytes and thrombocytes because they are not counted with the hemacytometer. However, hemacytometer counts are labor intensive, a greater potential for mathematical errors exists, and WBC distribution on stained blood smears may not be random.

One can estimate WBC counts from the stained blood smear by multiplying the average number of WBCs per field of view by a given factor (the factor is usually 1,500 for the 40× to 45× high-dry microscope objective or 2,000 for the 50× oil-immersion microscope objectives, respectively). Advantages of this technique include the stability of the blood film and the labor-efficient process of WBC count estimation. The major disadvantage is that the WBC count is estimated instead of quantitated.

Thus, estimated WBC counts may not be reproducible, poor smear technique may influence the estimate because of cell lysis or maldistribution. Coefficient of variation for hemacytometer-derived WBC counts (eosinophil Unopette method) are 12.7% as opposed to 28% for estimated WBC counts from stained blood smears.⁶⁸ However, the latter technique may be useful to indicate hematologic trends for which the lability or volume of the blood specimen would otherwise preclude hematologic study. Ultimately, the future of avian hematology depends on the development of semi-automated or automated techniques to determine avian WBC counts in a rapid, accurate, economical, and labor-efficient manner.

STAINED BLOOD SMEAR

The stained blood smear is used to perform the WBC differential cell count and morphologic examination of WBCs. Although considerable discussion has revolved around the best method to produce acceptable avian blood smears, both the wedge technique (with two glass slides) and coverslip technique (with two square glass coverslips) can be used. The major factors in producing high-quality smears are manual dexterity and practice.

It is generally assumed that WBCs are randomly distributed in blood smears, which may not be the case. Hematologic studies have shown that WBCs are more randomly distributed in coverslip smears than in wedge smears where large cells, such as monocytes, tend to be carried to the lateral margins and feathered edge of the smear. Furthermore, this problem is magnified with poor smear technique. Depending on the type of smear and the method of examination, WBC differential counts may vary. The accuracy of the WBC differential count can be increased by counting more cells. To increase the accuracy of the differential count significantly, one must perform a 400-cell count as opposed to 100 cell WBC differential count.

In summary, the smear quality directly influences the accuracy of the differential cell count and estimated WBC counts. Also, smears that are made at the time of blood specimen collection reflect the morphologic changes in WBCs at that point. If smears are made from the blood specimen at a later time, potential artifacts include increased numbers of smudge cells, distortion of WBCs and thrombocytes, vacuolation of monocytes, swelling of lymphocytes, and changes in WBC tinctorial properties.⁴³

LEUKOCYTE DIFFERENTIAL COUNT

White blood cell morphology may vary between species of birds (see Chapters 122–125). Examples include the appearance of granule morphology and cytoplasmic coloration of raptor eosinophils⁴ and the shape of heterophil granules in certain species of birds.⁷ With currently accepted criteria of WBC identification,

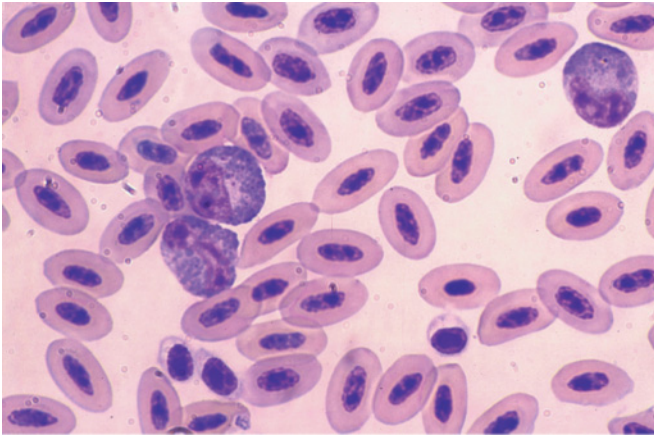


FIGURE 50.1 Blood smear from an African grey parrot. A left shift is indicated by the presence of immature heterophils (band, metamyelocyte, and myelocyte). All of these cells display mild toxic change (cytoplasmic basophilia).

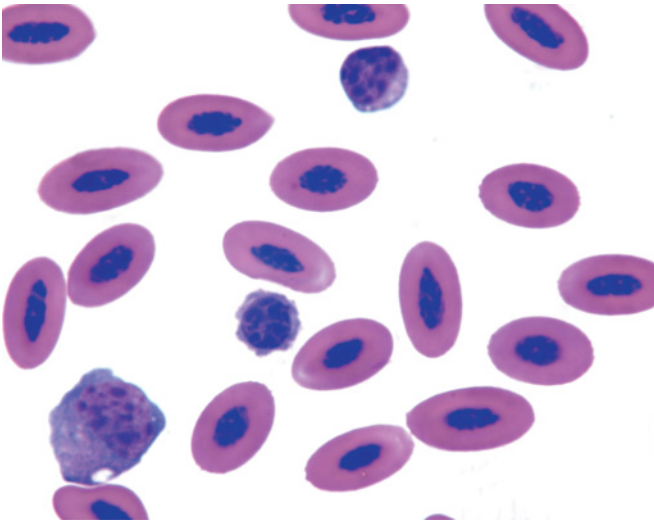


FIGURE 50.2 Blood smear from a great horned owl. A monocyte (lower left), thrombocyte (center), and lymphocyte (top) are present.

performance of the WBC differential count in healthy and sick birds may be challenging and requires practice. Problems may be encountered in identifying left shifts in the heterophil population⁴³ (Fig. 50.1) and in distinguishing some small lymphocytes from thrombocytes⁷³ (Fig. 50.2), monocytes from large lymphocytes (Fig. 50.3), reactive lymphocytes from rubricytes,⁵ heterophils from eosinophils^{21,42} (Fig. 50.4), and extremely toxic promyelocytes (mesomyelocytes) from basophils⁶⁸ (Fig. 50.5).

Detection of left shifts in avian blood is more difficult because avian heterophils have less nuclear segmentation than mammalian neutrophils.⁴³ Furthermore, heterophil granules tend to obscure nuclear morphology in Romanowsky-stained blood smears. With these preparations, only marked left shifts can be easily identified (Fig. 50.1). The presence of promyelocytes, that contain both basophilic and eosinophilic granules,²⁸

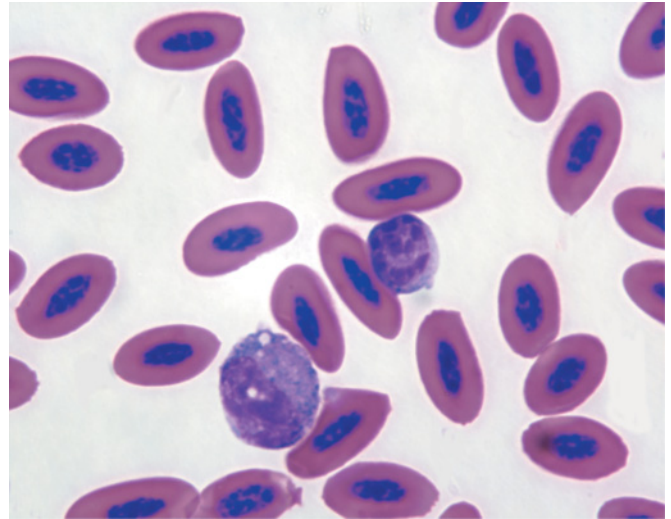


FIGURE 50.3 Blood smear from a great horned owl. A monocyte (lower left) and lymphocyte (upper right) are present.

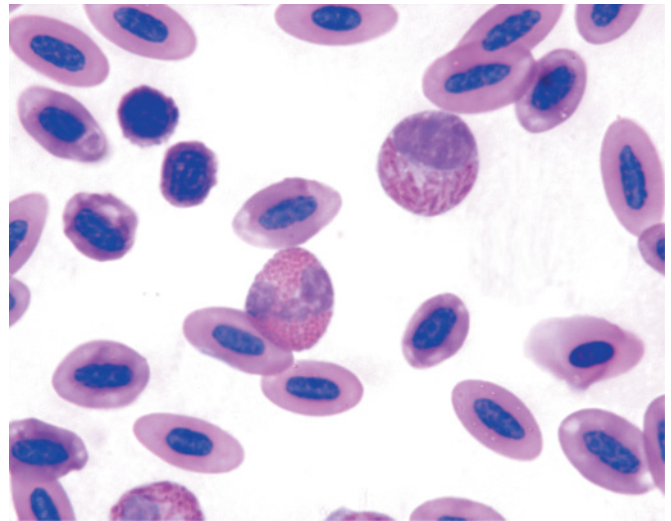


FIGURE 50.4 Blood smear from a red-tailed hawk. The myelocytic heterophil (top right) has needle-shaped, dull red granules, whereas the eosinophil (left) has round, bright, red-orange granules.

indicates an intense left shift⁴³ (Fig. 50.5). Subtle left shifts can be accurately quantitated by examination of hematoxylin-stained blood smears in which the nucleus is stained but the granules remain unstained.⁴³ This technique permits determination of a nuclear lobulation score, a sensitive indicator of a left shift.

CHANGES IN LEUKOCYTE MORPHOLOGY

Changes in leukocyte morphology are important observations that permit assessment of the severity of the disease process. Occasionally, morphologic changes in leukocytes may provide a definitive medical diagnosis.

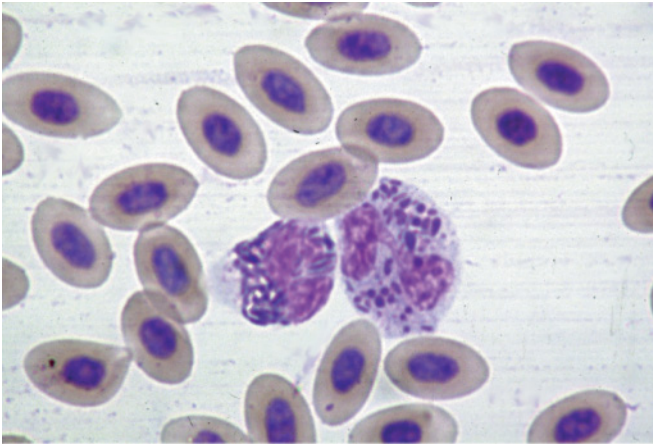


FIGURE 50.5 Blood smear from a chicken. Segmented heterophil and toxic heterophil promyelocyte. Note purple and red granules in the promyelocyte.

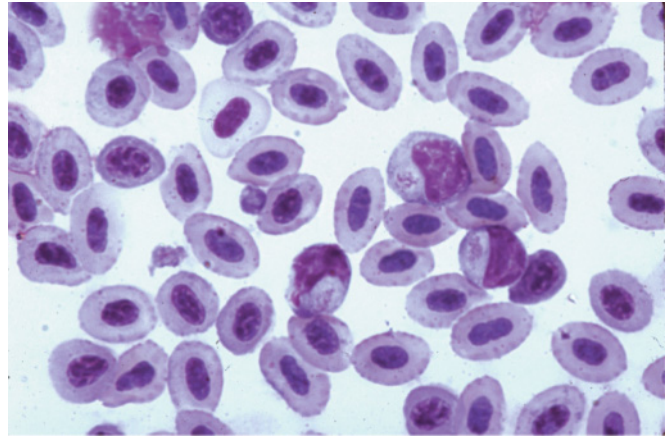


FIGURE 50.7 Blood smear from a Bali mynah that has disseminated toxoplasmosis. Note round to oval intracytoplasmic organisms in the mononuclear cells.

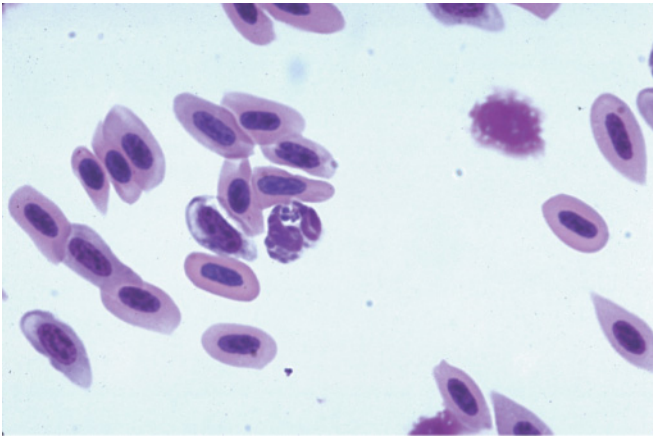


FIGURE 50.6 Blood smear from an Amazon parrot that has septicemia. Note the phagocytosed bacterial cocci within a heterophil.

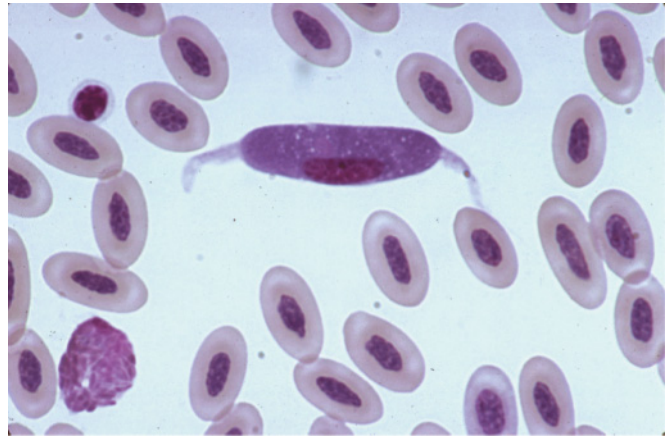


FIGURE 50.8 Blood smear from a great horned owl. *Leukocytozoon* sp. gametocyte is present in a leukocyte. The affected cell is enlarged, elongated, and has pointed cell margins.

Toxic Changes

Variable degrees of degranulation, cytoplasmic basophilia, cytoplasmic vacuolation, and cellular swelling constitute toxic changes in avian heterophils⁴⁰ (Figs. 50.1 and 50.5). Ultrastructurally, cellular swelling and cytoplasmic vacuolation are the result of intracellular edema. Degranulation is a sequel to dissolution of the granule matrix. Cytoplasmic basophilia is explained by the persistence of ribosomes. In Romanowsky-stained avian blood smears, cytoplasmic basophilia is the last manifestation of toxic change to disappear with convalescence.⁴⁰

Infectious Disease

Inclusions in avian WBCs are seen infrequently but may provide a definitive diagnosis when observed. Examples include phagocytosed bacteria in heterophils of septicemic birds (Fig. 50.6), *Atoxoplasma* sp. organisms in the

cytoplasm of monocytes (Fig. 50.7), *Leukocytozoon* sp. organisms in the cytoplasm of WBCs or erythrocytes (Fig. 50.8), and elementary bodies of *Chlamydophila psittaci* in the cytoplasm of various WBCs (Fig. 50.9).

After exposure to antigens and stimulation of the immune system, scattered lymphocytes may become larger (a prelude to blast transformation or plasmacytoid differentiation) and have dark blue granular cytoplasm. Such cells are classified as reactive lymphocytes morphologically. In lymphoid neoplasia, the majority of lymphocytes may appear immature or reactive.

The clinician also should be familiar with stain-induced changes in cellular morphology. Stain-induced artifacts, such as degranulation and intensified cytoplasmic coloration of heterophils in Diff-Quik®-stained preparations, should not be mistaken for toxic changes.⁴³ Also, partial heterophil degranulation may leave a round granule core, causing affected cells to resemble eosinophils.^{43,65} This condition is frequently seen with aqueous-based stains when short fixation times are

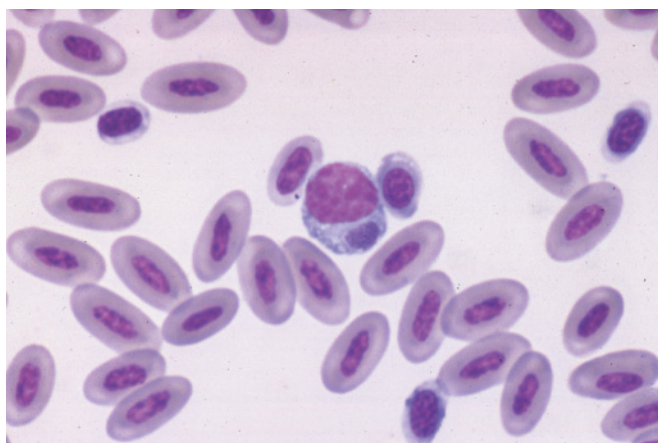


FIGURE 50.9 Blood smear from parrot that has chlamydiosis. Intracytoplasmic elementary bodies of *Chlamydoiphila psittaci* are present in a monocyte. (Courtesy of Dr. Michele Menard, Veterinary Cytopathology, Gresham, OR.)

used. Cytochemical staining has the potential to reliably distinguish heterophils from eosinophils for which cellular identification is uncertain.³

As avian hematology progresses, laboratories should undertake a coordinated effort to modernize avian leukocyte nomenclature, uniformly evaluate morphologic changes in blood cells, and consistently report semi-quantitative hematologic data. Standardized guidelines will facilitate comparison of hematologic data between laboratories.

REFERENCE INTERVALS

Reference intervals are used to identify laboratory test results or values that discriminate between health and disease (see Chapter 131). Establishment of meaningful reference intervals requires data collection from an adequate number of individuals constituting a precisely defined population. The population must be defined precisely because test values can be influenced by species of bird, age, sex, health and reproductive status, diet, and climate or environment. In addition, the method of specimen collection and analytical technique can influence test results. With these intervals, laboratory test values suggestive of health or disease may be distinguished more reliably.

Reference intervals for various species of companion and exotic birds have been published in journals and textbooks. Several of these publications are especially noteworthy based on the large numbers of individuals sampled, a defined reference population, and attention to age-related changes in hematologic data.^{19–23} Sometimes, avian clinicians are presented with ill patients that are rare exotic birds or juvenile birds of common species for which reliable reference intervals do not exist. In such cases, analysis of control blood samples from clinically healthy birds of the same species, age, sex, and environment permit identification of abnormal hematologic trends.^{29–31,74}

HEMATOPOIESIS WITH EMPHASIS ON LEUKOCYTE PRODUCTION

Primitive cells in the yolk sac initiate blood-cell development during the first few days of embryonic incubation. Early erythroblasts are recognizable by day 5 in the developing lung, and their appearance precedes vasculogenesis.⁴⁵ By 10–15 days of incubation, hemopoietic activity peaks in the yolk sac and is widespread in other tissues, including bone marrow, liver, kidney, spleen, thymus, bursa of Fabricius, aorta, heart, pharynx, cranial nerves, spinal ganglia, subcutaneous tissues, and muscles.⁴⁷ Although the bone marrow is the major site of hemopoietic activity post-hatching, foci of hematoipoiesis also may be observed in the liver, spleen, and kidney of companion birds.⁶⁹ In birds, extramedullary hematoipoiesis is widespread and involves granulopoiesis, wherein the majority of the cells contain eosinophilic granules and nuclei in various stages of lobulation. Granulopoiesis is especially prominent in the liver, kidney, and spleen, but it may involve the heart and subdural spaces. Granulopoiesis, especially in the spleen, bursa, and thymus, involves production of heterophils.⁴⁷

In bird bone marrow, granulopoiesis occurs in the extravascular spaces, whereas erythropoiesis and thrombopoiesis occur in the bone marrow vascular sinuses. Sites of hematoipoiesis in birds involve non-pneumatized long bones and the axial skeleton. A practical example is acquisition of bone marrow aspirates from the tibia because the humerus and femur frequently are pneumatized. During early life, hematoipoiesis is distributed throughout the skeleton, except for the skull.^{63,70}

Lymphocyte production occurs in primary (thymus, bursa of Fabricius) and secondary (spleen; gut-associated lymphoid tissue, including cecal tonsils; bronchial-associated lymphoid tissues; paraocular tissues, including conjunctival-associated lymphoid tissue; paranasal sinuses, and miscellaneous lymphoid follicles along lymphatic vessels) extramedullary tissues. Lymphocytes are present in the thymus and bursa of the embryo by 10–14 days of incubation. As lymphopoiesis progresses, the secondary lymphoid tissues produce the majority of the lymphocytes, whereas the primary lymphoid tissues (thymus and bursa) involute with sexual maturity.

LEUKOCYTE FUNCTION AND RESPONSE IN HEALTH AND DISEASE

Interpretation of avian leukograms should be based on absolute leukocyte values per microliter of blood. This results in fewer erroneous interpretations compared to reliance on relative percentages alone.³⁹ Although diurnal rhythms of leukocyte counts have been demonstrated occasionally in birds (e.g. chickens),³⁵ these fluctuations have little effect on leukogram interpretations in a clinical setting.

Few scientifically-controlled studies have been performed to evaluate avian WBC responses.⁴³ Therefore, interpretation of avian leukograms has relied on extrapolations from the veterinary literature and individual clinical experience. Although hematologic studies of patients with spontaneous disease have increased our knowledge of avian hematology, precise interpretation of avian WBC responses is obscured by numerous variables and incomplete patient data concerning sample collection, method of analysis, restraint, drug administration, etc. The following discussion is intended to provide basic guidelines for leukogram interpretation.

HETEROPHIL OR LYMPHOCYTE PREDOMINANCE IN THE BLOOD IN STATES OF RELATIVE HEALTH

Birds are similar to rodents in that the primary circulating WBC in some species is the heterophil, whereas in other species the lymphocyte predominates. Examples of birds that have a predominance of heterophils include greater sulfur-crested cockatoos, herring gulls, hyacinth macaws, rainbow lorikeets, yellow-crowned Amazon parrots, African grey parrots, and pigeons. Examples of birds that have a predominance of lymphocytes include budgerigars, canaries, cockatiels, finches, rose-ringed parakeets, and many species of Amazon parrots. Hematologic studies of some species of adult birds, such as flamingos and white-naped cranes, are contradictory in that either heterophils or lymphocytes have been identified as the major circulating leukocyte in health. Possible explanations for these divergent observations include stress of capture, handling, caging, social interactions, and environmental conditions. These variables, singly or in combination, could induce heterophilia or lymphopenia, resulting in aberrant reference intervals.

Hematologic study of neonatal and juvenile birds indicates that heterophilic leukocytosis can be observed with some frequency.¹⁵⁻¹⁷ The presumed mechanism for this observation is stress. Furthermore, some birds that have a predominance of lymphocytes in the adult leukogram frequently have a predominance of heterophils during the post-hatch and juvenile periods. Transition to the normal adult leukogram eventually occurs with age (generally 8–12 weeks of age), a similar process typically occurs in young ruminants by weaning age. Total WBC counts frequently are elevated post-hatch (a stressful period of neonatal life) and decrease with age.

From the discussion and examples given above, one can readily appreciate the need for reference intervals so that leukograms from birds of various species and ages are interpreted correctly. In the case of expensive or rare birds, an annual physical examination and CBC provide individual reference data to detect subtle changes in individual health status. This procedure is expensive, but has proven to be effective in human health care.

LEUKOCYTOSIS AND LEUKOPENIA

Leukocytosis

Leukocytosis, is frequently the result of physiologic processes, infection, or inflammation. Physiologic leukocytosis is precipitated by excitement, fear, forced flight, and excessive muscular activity. Increases in heterophils or lymphocytes may account for the leukocytosis. If the mechanism in birds mimics that of mammals, leukocytosis could be related to epinephrine release or muscular exertion, both of which cause increased cardiac output, increased blood pressure, and a washout of leukocytes from the microvasculature into the mainstream of circulation.

In inflammatory and infectious conditions of birds, heterophilia frequently is observed and the degree of heterophilia may be more pronounced than comparable neutrophilia in mammals.¹³ Examples include acute chlamydiosis, mycobacteriosis, and disseminated mycosis where the total WBC count may exceed 100,000 cells/ μL of blood. In the case of birds that have a predominance of lymphocytes such as chickens, a heterophil-lymphocyte reversal may be observed.^{11,43} In some infectious conditions of birds, a lymphocytosis may be noted. An example is chronic chlamydiosis where the immune system has been stimulated as observed by plasmacytosis, which is most notable in the liver and spleen.

Leukopenia

Leukopenia in birds that have a predominance of heterophils usually is the result of heteropenia. In birds that have a predominance of lymphocytes, leukopenia often is synonymous with lymphopenia. Causes of leukopenia vary, depending on whether heterophils, lymphocytes, or both cell lines are affected. Specific causes of heteropenia and lymphopenia, including those mechanisms resulting in leukopenia, are discussed in detail below.

HETEROPHIL RESPONSES

Heterophils provide a first line of defense against infection (Table 50.1). Avian heterophils and mammalian neutrophils differ both morphologically and biochemically. Morphologically, avian heterophils are identified by the presence of large, dull red, frequently needle-shaped granules (Fig. 50.4). The presence of these eosin-staining granules has given rise to the term heterophil (*heteros* from the Greek meaning different), as opposed to the mammalian neutrophil whose granules are generally neutral in Romanowsky-stained blood smears. A left shift exists when excess band heterophils are present in the blood. Because avian heterophils are less segmented in health compared with mammalian neutrophils, identification of left shifts is more challenging in avian species.⁴³

TABLE 50.1 Causes of Heterophilia and Heteropenia in Birds

Heterophilia	
Physiologic response	
Infection	Bacteria, viruses, fungi, parasites
Tissue destruction or necrosis	Thrombosis and infarction, inflammation
Drug administration	Corticosteroids, estrogen
Miscellaneous	Acute, severe stress, foreign bodies, hemorrhage or hemolytic disease
Heteropenia	
Infection	Overwhelming bacterial infection, viral-induced hemopoietic cell destruction
Drug administration	
Predictable	Cyclophosphamide, progesterone
Idiosyncratic	Piperacillin or doxycycline
Neoplasia	Leukemia, metastatic disease (disseminated lymphosarcoma), radiation exposure

Toxic changes of heterophils include cytoplasmic basophilia, cytoplasmic vacuolation, variable degranulation, and cellular swelling (Figs. 50.1 and 50.5). Toxic degranulation must be distinguished from stain-induced degranulation. Toxic degranulation is the result of severe inflammation or infection and is associated with cytoplasmic basophilia. Stain-induced degranulation results from partial or complete granule dissolution when heterophils on the blood smear are exposed to aqueous-based stains (such as Diff-Quik® stain) with a short fixation time.⁴³

Biochemically, avian heterophils lack myeloperoxidase, an enzyme that is largely responsible for the efficient oxidative bactericidal activity of mammalian neutrophils.^{3,62} A measurable oxidative burst has been observed for avian heterophils, but it is insignificant in bacterial killing. Avian heterophils accomplish effective nonoxidative bacterial killing by myeloperoxidase-independent methods with granule-derived proteins.⁶² These substances include lysozyme, acid phosphatase, cathepsin, and β -glucuronidase (hydrolase) activities, and cationic proteins.^{18,19} The cationic proteins include β -defensins (e.g. gallinacins, chicken heterophil antimicrobial peptides, and turkey heterophil antimicrobial peptides), which are natural antimicrobials.⁷¹ In addition to bacteria, avian heterophils have been shown to kill yeast (e.g. *Candida albicans*).

Avian heterophil function (adherence, chemotaxis, phagocytosis, and bacterial killing) is fairly efficient in host defense against bacteria in conjunction with the humoral immune system.^{4,5,6,38} Avian heterophil functions are less well understood than mammalian neutrophil function and constitute an area for future research.

Physiologic Heterophilia

One can observe physiologic heterophilia after excitement, fear, or short-term strenuous exercise (the fright

or flight response). The heterophilia occurs rapidly but is transient. The presumed mechanism, based on studies in other species, is an epinephrine- or exercise-mediated increase in microvascular blood flow that shifts marginated heterophils into the mainstream of circulation. Concurrent lymphocytosis also may be observed and may overshadow the heterophilia, especially in those species that have higher lymphocyte counts in health. Because of the frequent occurrence of physiologic leukocytosis, it is wise to obtain hematologic specimens before the patient is unduly perturbed. Physiologic responses are frequently confused with stress leukograms where lymphopenia is expected.²⁰

Corticosteroid-induced Heterophilia

Corticosteroid-induced heterophilia is observed sporadically in diseased or severely stressed birds and is the result of corticosterone release from the adrenal cortex. An example of developing heterophilia in response to forced confinement has been reported in captive herring gulls.^{2,7} Corticosteroid-induced heterophilia is observed more frequently with exogenous corticosteroid administration or injection of adrenocorticotropic hormone (ACTH), which stimulates endogenous corticosterone release. The phenomenon is dosage and route dependent.¹ Developing or concurrent lymphopenia separates this response from physiologic leukocytosis.

Inflammation- or Infection-induced Heterophilia

Heterophilia is frequently observed in conjunction with tissue damage induced by inflammation or bacterial infection (including chlamydiosis).^{2,7,43} Occasionally, the source of tissue damage provoking heterophilia may be obscure. Examples include hemorrhage, oxidant-induced RBC destruction, and lead poisoning.^{34,41,46} Experimental studies have shown that significant heterophilia can occur within 6 hours of induced inflammation and that cell counts peak 4-fold greater than baseline values by 12 hours post-inflammation.⁴³ As bone marrow reserves of segmented heterophils are depleted, a left shift accompanied by toxic changes of heterophils may appear within 24 hours. The presence of promyelocytes (cells with round nuclei and a mixture of purple and red cytoplasmic granules) indicates an intense shift.⁴³ As the bone marrow responds to tissue demands for heterophils, the leukocytosis and heterophilia intensify. A return toward baseline values with disappearance of the left shift and toxic change indicates convalescence. In companion birds, total leukocyte counts may exceed 100,000 cells/ μ L in chronic chlamydiosis and mycobacteriosis, especially in gray cheek parakeets and macaws.

Heteropenia

The three most common mechanisms for production of neutropenia in mammals include deficient neutrophil production in the bone marrow, a shift in neutrophils

from the circulating to marginal cell pool, and emigration of neutrophils from the blood into the tissues at a rate that exceeds neutrophil replacement from the bone marrow.³⁹ Similar mechanisms undoubtedly exist in birds but have not received detailed study. However, anecdotal reports in the literature and personal observations tend to support these mechanisms.

Any instance of heteropenia suggests a guarded prognosis until favorable resolution of the condition can be ascertained by additional hematologic study.

Deficient Heterophil Production

Deficient heterophil production can be the result of hemopoietic stem cell destruction by infectious agents, drugs, or ionizing radiation and myelophthisis-associated loss of hemopoietic space.

Leukopenia and heteropenia have been reported in pet birds due to viral infections (herpesvirus, polyomavirus, and psittacine reovirus); however, such reports largely represent undocumented clinical or laboratory impressions.^{24,25,66} Drug-induced myelosuppression may be predictable, as observed in cyclophosphamide-treated chickens and turkeys,²⁴ or may be an idiosyncratic adverse drug reaction, as observed in a budgerigar after antibiotic (piperacillin or doxycycline) treatment.⁶⁷ Toxic change of heterophils and vacuolation of hemopoietic precursor cells may be observed with drug toxicity.

Myelophthisis-associated loss of hemopoietic space is observed infrequently in clinical practice. In our experience, this cause of heteropenia has been associated with disseminated lymphoid neoplasia wherein proliferating neoplastic lymphocytes replace normal hemopoietic cells.

Heterophil Shifts from the Circulating to Marginal Pool

In mammals, early endotoxemia or Gram-negative sepsis results in a shift of neutrophils from the circulating to marginal cell pools where they are not quantitated by the WBC count. The neutropenia is transient (1–3 hours duration) and is followed by a rebound leukocytosis if the patient survives. This mechanism may occur in birds but is not well documented.

Severe Tissue Demands for Heterophils

Overwhelming tissue demand for heterophils at a rate that exceeds cell replacement by the bone marrow also produces heteropenia. Examples include acute severe peritonitis and necrotizing enteritis. Heteropenia may be accompanied by a degenerative left shift and toxic change of heterophils.

Lymphocyte Responses

As mentioned previously, lymphocytes may be the major circulating leukocyte in the blood of some avian

species (Figs. 50.2 and 50.3). Characterization of the blood lymphocyte population in mammals indicates that the majority of these cells are small, mature, long-lived, memory T cells. This probably is the case in avian species. For example, chicken blood lymphocytes have shown to be approximately 14% B cells and 72% T cells by direct and indirect immunofluorescence, respectively.⁶⁰ The remaining blood lymphocytes (approximately 14%) are probably natural killer or null cells.

Lymphocytosis

Physiologic lymphocytosis represents a transient phenomenon in birds after excitement, fright, or struggling during venipuncture (Table 50.2). Lymphocytosis may overshadow any heterophilia. This response may be especially noticeable in healthy birds that have high circulating lymphocyte counts, and may be more prominent in young birds (Fig. 50.10).

TABLE 50.2 Causes of Lymphocytosis and Lymphopenia in Birds

Lymphocytosis	
Physiologic response	
Antigenic stimulation	Chronic viral infection, chronic bacterial infection, chronic fungal infection, parasitic diseases
Lymphoid neoplasia	Lymphosarcoma, lymphocytic leukemia
Lymphopenia	
Acute systemic infection	
Corticosteroid-induced	Exogenous corticosteroid administration Endogenous corticosterone release (acute, severe stress)
Immunosuppression	Immunosuppressive drugs, radiation

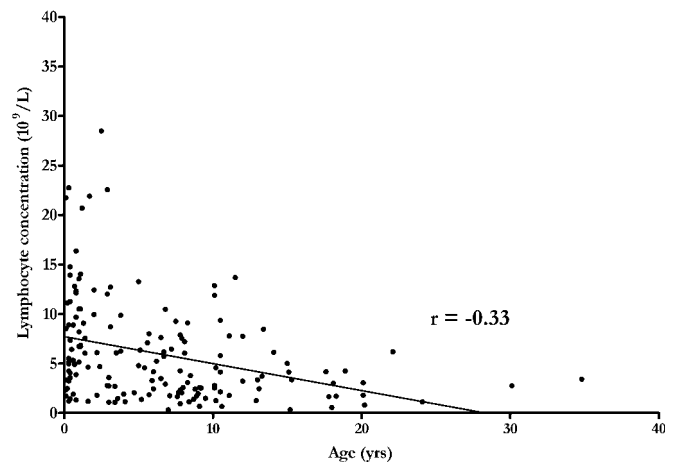


FIGURE 50.10 Relationship of the lymphocyte count with age in 169 blood samples from clinically healthy psittacine birds. Relatively high lymphocyte counts were common in birds less than 4 years of age.

Lymphocytosis secondary to antigenic stimulation is frequently observed in birds that have chronic infectious (bacterial, viral, fungal, or parasitic) or inflammatory diseases wherein the blood lymphocyte pool is expanded because of persistent antigen exposure, most notably in chronic bacterial or viral diseases.⁴³ Occasionally, the lymphocytosis is extreme as in a crane that had granulomatous disease and an absolute lymphocyte count of 45,900 cells/ μ L.

Lymphoid neoplasia such as lymphosarcoma with a leukemic blood picture^{27,58} (Fig. 50.11), or lymphoid leukemia (Fig. 50.12), may be associated with lymphocytosis. In our experience, lymphoid leukemias produce extreme elevations in the absolute lymphocyte count (as much as 200,000 lymphocytes/ μ L).

Lymphopenia

Lymphopenia is frequently the result of severe stress-induced endogenous corticosterone release or

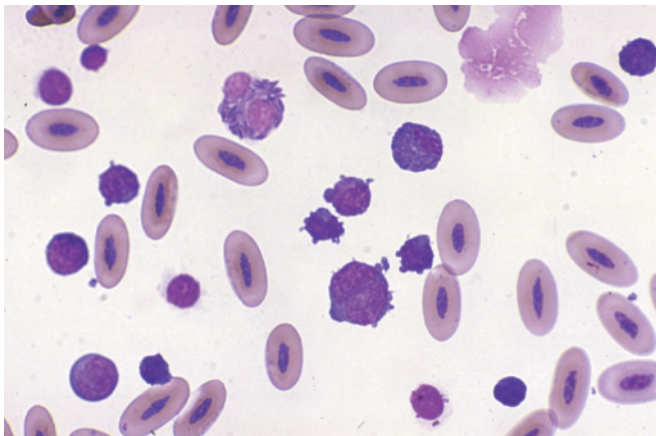


FIGURE 50.11 Blood smear from an emu that has disseminated lymphoma. Immature, neoplastic lymphocytes within the blood smear have dark blue cytoplasm with broad pseudopodia.

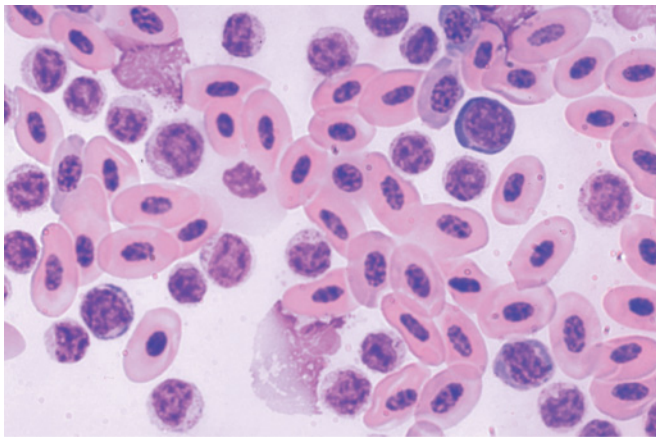


FIGURE 50.12 Blood smear from a cockatoo that has lymphocytic leukemia. The leukocytes are predominately mature, well-differentiated lymphocytes.

exogenous corticosteroid administration (Table 50.2). Corticosteroid administration produces a rapid, predictable, transient redistribution of mammalian T cells to the bone marrow or other tissue compartments, resulting in lymphopenia.¹ Studies in chickens have documented a rapidly developing but transient lymphopenia after corticosteroid administration. Stress-associated lymphopenia is more variable and often difficult to document hematologically in apparently clinically stressed birds. Heterophilia and concurrent lymphopenia are the hallmarks of a stress leukogram and may be observed in diseased or severely stressed birds, such as those subjected to forced molting through severe feed restriction.³²

Lymphopenia of acute infection may have a complex origin, involving one or more mechanisms. These mechanisms include endogenous corticosterone release with temporary lymphocyte redistribution, temporary trapping of recirculating lymphocytes within lymphoid tissues to promote antigen contact, and direct destruction of lymphoid tissue, especially during viral infection.³⁹

Last, drug- or toxin-induced lymphopenia may be observed infrequently. Examples include cyclophosphamide administration in chickens and turkeys, and crude oil ingestion by gulls.

MONOCYTE RESPONSES

The monocyte-macrophage system is composed of stem cells, monoblasts, and promonocytes in the bone marrow; monocytes in the bone marrow and blood; and macrophages within various body tissues. Studies in turkeys indicate that phagocytic uptake of blood particulate material occurs predominantly by macrophages in the liver, spleen, and bone marrow.⁵⁵

Monocytes are produced in the bone marrow and released into the blood at an early age compared with heterophils. A bone marrow storage pool of monocytes apparently does not exist. Once released into the blood, monocytes circulate for a short time and emigrate from the blood vessels into the tissues (Fig. 50.3 and Fig. 50.13). Those monocytes that mature into tissue macrophages may have an extended lifespan ranging from days to months.

Tissue demands for macrophages are met primarily by recruiting monocytes from the blood and increasing monocyte production in the bone marrow.⁶⁰ Macrophages can undergo limited mitosis in situ, but such activity generally accounts for less than 5% of the total macrophage population.

Monocytes are generally the largest circulating leukocyte in avian blood in health and typically account for 1–3% of the circulating WBC population. These cells have round to oval nuclei, slightly condensed chromatin, gray cytoplasm, and occasional pseudopodia. Cytoplasmic vacuolation also may be observed, but is more prominent if the blood stands for a while before smears are made. Difficulty can be encountered in distinguishing monocytes from large lymphocytes in some

birds. In such cases, cytochemistry can be used for definitive cell identification.⁶¹

Monocytosis

Monocytosis frequently is associated with chronic diseases such as granulomatous lesions, nonspecific tissue necrosis, bacterial infections (e.g. mycobacteriosis and chlamydiosis), deep mycosis (e.g. aspergillosis, parasitism), and zinc-deficient diets (Table 50.3).^{26,76} A frequent misconception among avian clinicians, however, is that monocytosis is only seen in chronic diseases and, when present, is indicative of granulomatous inflammation. In fact, monocytosis can be observed in both acute and chronic diseases. Experimental studies in chickens have shown that significant monocytosis can be observed in the blood within 12 hours after induced inflammation or bacterial airsacculitis, with peak monocyte counts occurring within 24 to 48 hours.^{14,43}

Monocytopenia

Monocytopenia is clinically unimportant and difficult to document because of the wide range in avian monocyte counts. In instances of pancytopenia, such as viral-induced hematopoietic cell damage, heteropenia deserves more clinical attention because of the possibility of secondary infections.

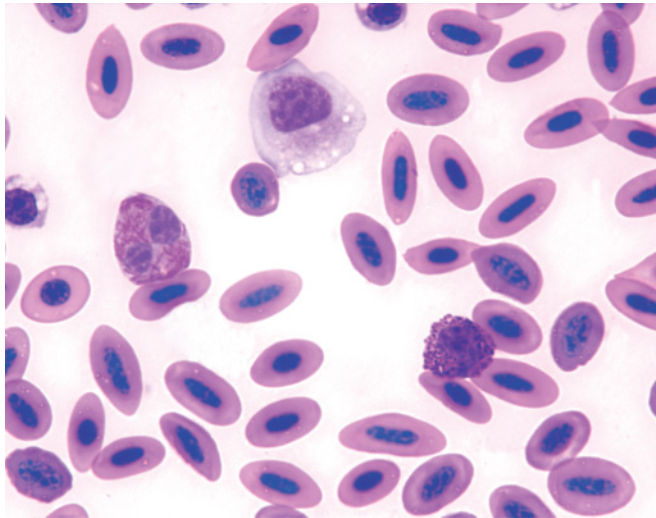


FIGURE 50.13 Blood smear from a red-tailed hawk. A heterophil, monocyte, and basophil are present.

TABLE 50.3 Causes of Monocytosis in Birds	
Granulomatous inflammation	
Acute inflammation	
Bacterial infection	Mycobacteriosis Chlamydiosis (chronic)
Fungal infection	Aspergillosis
Parasitism	
Zinc-deficient diets	

BASOPHIL RESPONSES

Avian basophils are recognized by their round to oval nucleus and prominent, round, purple granules in Romanowsky-stained blood smears (Fig. 50.13). In healthy birds, basophils usually are more numerous in the blood than are eosinophils. This observation has been made in many avian species.

Functions of avian basophils appear to be similar to those of mammalian basophils (see Chapter 44) but investigations of cell function are sparse or anecdotal. The involvement of avian basophils in acute inflammation is well documented. In chickens, basophilic infiltration of tissues has been observed with early (1.5–3 hours) inflammation of skin, wattle, skeletal muscle, and mesentery.^{12,54,72} Involvement of basophils has also been demonstrated in avian cutaneous hypersensitivity and systemic anaphylaxis.⁵² Clinical and experimental evidence also suggests that avian basophils are involved in the host response to internal and external parasites, including schistosomes, soft-bodied ticks, and air sac mites. Evidence also exists for basophil-associated tumor cytotoxicity wherein intense basophilic infiltrates have been observed in experimentally induced Rous sarcomas in chicken wing webs. In contrast to mammalian basophils, avian basophils also are actively phagocytic.³⁷

Basophilia

Generally, only sustained overt basophilia can be detected by routine hematologic methods wherein basophils constitute at least 3–6% of the leukocyte differential cell count. Although absolute basophil counts, determined using a hemacytometer and toluidine blue diluent, have been performed in birds,⁵² reports of blood basophil responses in disease are sporadic (Table 50.4).

Basophilia is reported to occur with respiratory diseases and severe tissue damage in pet birds.⁵⁹ This finding is partially corroborated by a trend toward development of basophilia in chickens that have experimentally-induced salmonellosis wherein basophil counts tended to increase within the first week of infection. Although the mechanism of basophilia is unknown, it may be related to the proinflammatory response observed in humans.⁶⁵ Blood basophilia also has been

TABLE 50.4 Causes of Basophilia in Birds

Tissue damage or perturbation	Acute inflammation (skin, wattle, muscle, mesentery) Severe nonspecific tissue damage Bacterial infection Respiratory disease Systemic anaphylaxis
Stress	Feed restriction, starvation Forced molting
Parasitism	Schistosomes, ticks, air sac mites
Miscellaneous	Ingestion of mycotoxin-contaminated feed

observed with internal and external parasitism. Examples include experimental infection of chickens that have *Austrobilharzia variglandis*, and natural infestation of canaries and finches with air sac mites. Basophilia can occur alone or in conjunction with eosinophilia. Basophilia also has been observed in chickens^{49,53} and ducks after significant feed restriction, and is presumed to be a harbinger of severe stress. Apparently birds differ from mammals in that basophilia occurs in stress and that blood basophil counts apparently are unaffected by corticosteroid administration.⁴⁹ Basophilia also has been reported in chickens, presumably in response to mycotoxin-contaminated feed.⁵⁰

Basopenia

Basopenia cannot be detected reliably without performing absolute basophil counts. The presence of basopenia in other species is of limited clinical importance in routine health care, as may be the case in avian patients.

EOSINOPHIL RESPONSES

Avian eosinophils are recognized by their round, often red-orange granules, and light blue cytoplasm in Romanowsky-stained blood smears. However, raptor eosinophil granules may occasionally be rod shaped, and granule tinctorial properties may vary slightly between species. Eosinophils generally are the least frequently encountered leukocyte in avian blood smears, with the exception of raptors in which these cells may account for 15% of the leukocyte differential cell count. In some species of birds, such as Amazon parrots, a leukocyte with round, colorless to light blue granules may be observed in the stained blood smear. The common practice is to identify such cells as eosinophils because typical eosinophils with red-orange granules have not been observed. Ultimate identification of this unusual leukocyte will require both ultrastructural and cytochemical study.

Eosinophilia

Avian species are similar to horses in that eosinophilia is observed infrequently in species other than raptors, although parasitism is observed frequently (Table 50.5). Eosinophilia has been observed in chickens that have

facial edema; chickens experimentally infected with schistosomes or naturally infected with *Trichostrongylus tenuis*; and in quails, chickens, and ducks injected intraperitoneally with horse serum or bovine serum albumin. The magnitude of the eosinophilia ranges from 6% to 56% of the leukocyte differential count in both experimental and natural disease. Furthermore, lymphocyte-eosinophil interaction has been demonstrated indirectly. Marked secondary eosinophilia has been observed in chickens and ducks after repeated antigen administration.⁴⁸ Irradiation has been shown to depress development of eosinophilia in chickens, whereas thymectomy had little effect.⁵¹ The reason for this apparent paradoxical observation is that total-body irradiation affects all lymphocytes and it is difficult to remove all thymic tissue.

Eosinopenia

Eosinopenia is best defined by clinical experience. Because eosinophils are infrequently encountered in avian blood except for raptors, absolute eosinophil counts are necessary for a quantitative study of avian eosinopenia. A problem is readily apparent in that it may be difficult to distinguish eosinophils from heterophils in absolute counts unless cytochemistry is used.

In mammals, eosinopenia is observed after corticosteroid or ACTH administration. Single-dose administration results in sequestration of eosinophils and delayed eosinophil release from the bone marrow. Prolonged high-dose corticosteroid treatment causes decreased eosinophil production.

Eosinophil responses in sick birds are seldom mentioned, although indication of eosinopenia sometimes is apparent. Injection of long-acting ACTH into immature chickens has shown a suggestion of slight eosinopenia, but routine hematologic methods lacked the sensitivity needed for adequate quantitation of the cellular response. In summary, clarification of avian eosinophil responses in blood relies on evaluation of absolute cell counts. In addition, the importance of avian eosinophil responses within the tissues will only be clarified by immunohistochemical staining to reliably distinguish eosinophils from heterophils.

REMARKS ON PROGNOSIS AND HEMATOLOGIC TRENDS

Heterophil:Lymphocyte Ratios

Introduced as an indicator of stress in chickens, the heterophil:lymphocyte (H:L) ratio has been calculated on the basis of both absolute heterophil and lymphocyte counts or on relative percentages of these WBCs from the stained blood smear. When interpreting H:L ratios, one must remember that the ratio can increase with absolute heterophilia when the lymphocyte count is in the reference interval or with absolute lymphopenia when heterophil counts are in the reference interval. The true stress response, that is observed after

TABLE 50.5 Causes of Eosinophilia and Eosinopenia in Birds

Eosinophilia	
Facial edema	
Parasitism	
Exposure to foreign antigens	Horse serum, bovine serum albumin
Eosinopenia	
Severe stress	
Corticosteroid administration	

corticosteroid administration, is absolute heterophilia in conjunction with developing lymphopenia. In an experiment on feed restriction, ducks showed no change in H:L ratios; however, H:L ratios were increased in chickens and turkeys. In this study, it was felt that developing basophilia in chickens, turkeys, and ducks was a more uniform hematologic change denoting severe stress. In our opinion, H:L ratios currently represent another mathematical manipulation of limited clinical value in assessing stress in birds.

Unfavorable Prognostic Features

A guarded prognosis is suggested with heteropenia of any cause, especially if a left shift and toxic changes of heterophils are observed. If a left shift intensifies or becomes degenerative (where band heterophils and younger forms outnumber segmented heterophils), a guarded prognosis is suggested. Extreme leukocytosis also suggests a guarded prognosis until chlamydiosis, mycobacteriosis, deep mycosis, and leukemia are excluded.

Favorable Hematologic Trends

Hematologic trends in birds that are suggestive of recovery include resolution of extreme leukocytosis, leukopenia, heteropenia, left shift, toxic changes of heterophils, and lymphopenia.

NEW TECHNIQUES FOR ASSESSMENT OF AVIAN LEUKOCYTES

A flow cytometric technique for enumeration of quail, chicken and goose WBCs has been described.^{76,77} The technique relies on the differential affinity of avian RBCs and WBC types for fluorescent lipophilic dyes such as 3,3-dihexyloxycarbocyanine [(DiOC₆(3)], permitting cell separation based on fluorescence.^{56,75} This technique holds promise for application in other avian species because antibodies specific for cell surface markers are not required; however, a flow cytometer capable of fluorescence detection is necessary. Classification of avian lymphocytes into subtypes of T cells and B cells,¹ and distinction of thrombocytes from lymphocytes with antibodies specific for chicken thrombocytes,⁸ are established research techniques, but their incorporation into routine hematology is limited by the need for species-specific antibodies to WBC antigens and need of a flow cytometer.

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Biology of Lymphocytes and Plasma Cells

MICHAEL J. DAY

The Innate and Adaptive Immune Response

T Cells

Definition and Development

T Cell Activation

T cell functions

Function of CD4+ T cells

Function of CD8+ T cells

Function of $\gamma\delta$ T cells

NKT cells

Natural Killer Cells

B Cells and Plasma Cells

Acronyms and Abbreviations

ADCC, antibody-dependent cell-mediated cytotoxicity; APC, antigen presenting cell; BCR, B cell receptor; CD, cluster of differentiation; CTLA-4, cytotoxic T lymphocyte antigen 4; DAMP, damage-associated molecular pattern; Foxp3, forkhead box P3; GITR, glucocorticoid-induced TNF-receptor regulated gene; HEV, high endothelial venule; IFN, interferon; Ig, immunoglobulin; IL, interleukin; LGL, large granular lymphocyte; MAMP, microbe-associated molecular pattern; MHC, major histocompatibility complex; NF κ B, nuclear factor kappa B; NK, natural killer; NOD, nucleotide-binding oligomerisation domain; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; RIG, retinoic-acid-inducible gene; SCID, severe combined immunodeficiency; SmIg, surface membrane immunoglobulin; STAT, signal transducer and activator of transcription; TCR, T cell receptor; Th, T helper cell; TGF, transforming growth factor; TNF, tumor necrosis factor; Tr or T-reg, T regulatory cell.

The lymphoid cells are key components of the immune system. An increasing number of lymphoid subsets are now defined (Table 51.1). Some have roles in the innate immune response (natural killer [NK] cells and T cells expressing the $\gamma\delta$ T cell receptor [TCR]), but most are responsible for mediating adaptive immunity. These latter cells regulate the production of antibody (humoral immunity) or are responsible for cell-mediated immune effects such as cytotoxicity or delayed-type hypersensitivity. The fundamental importance of lymphoid cells is clearly demonstrated in animals with genetic inability to produce such cells (e.g. severe combined immunodeficiency; SCID) that are profoundly immunodeficient and readily succumb to infectious disease.^{34,36}

Lymphocyte subsets may be defined:

- phenotypically, by the expression of surface molecules
- anatomically, by their developmental pathways and distribution within lymphoid tissue
- functionally, by their end-effects in an immune response which relates to expression of particular membrane molecules and release of specific cytokines.

Lymphocyte biology is best defined in humans and rodents, but most features are highly conserved and have parallels in veterinary species.

THE INNATE AND ADAPTIVE IMMUNE RESPONSE

The innate immune response encompasses an evolutionarily older, more simplistic form of immunity that is continually present to provide immediate first-line defense from potential pathogens.²⁵ As the encounter with pathogens is most likely to occur at mucocutaneous surfaces, these are the anatomical regions that are particularly enriched for components of the innate immune system. A key part of the innate response is the presence of an epithelial barrier that has specific regional adaptations to enhance its effectiveness (e.g. the mucociliary escalator of the upper respiratory tract, keratinization of the cutaneous squamous epidermis). Mucocutaneous surfaces also frequently are bathed in antimicrobial secretions that contain a cocktail of enzymes (e.g. lysozyme), small antibacterial molecules

TABLE 51.1 Lymphocyte Subsets

Cell Type	Phenotypic Subtype	Functional Subtype	Function
B cell			Plasma cell precursor
Plasma cell			Immunoglobulin secretion
T cell	$\alpha\beta$ TCR+ CD4+	Th0	Precursor to functional CD4+ T cell subsets described below
		Th1	Cell-mediated immunity; limited help for antibody production. Mediates "type 1" immune response
		Th2	Help for antibody production, particularly IgE, IgG subclass and IgA. Mediates "type 2" immune response
		Th3	Regulatory T cell mediates oral tolerance
		Th17	Mediates proinflammatory immunopathology, response to infections and autoimmunity
		Treg	CD25+ CD4+ "natural suppressor" cell. Normally present to control autoimmune and allergic responses
		Tr1	IL-10 producing "induced suppressor" cell stimulated during immune response to exogenous antigen
	$\gamma\delta$ TCR+ CD4+		Predominantly surface intraepithelial population important in first-line defence to bacterial pathogens. May be type 1 and 2 subsets
NKT cell	$\alpha\beta$ TCR+ CD8+		Cytotoxic T cells. May be type 1 and 2 subsets
NK cell	Types I and II		Recognize lipid antigen expressed by CD1d Cytotoxic cell; performs antibody-dependent cell-mediated cytotoxicity (ADCC)

(e.g. defensins, alternative pathway complement molecules) and low-specificity polyreactive antibodies of the immunoglobulin (Ig) A and IgM classes. Within, and immediately beneath, the epithelial barrier are populations of innate immune cells including phagocytic cells (e.g. neutrophils, macrophages), NK cells and $\gamma\delta$ TCR+ T cells.

Another important leukocyte involved in innate immunity is the dendritic cell. In recent years, this cell has become the focus of intense interest with the recognition that it provides the key link between the innate and adaptive immune systems.^{16,20} The dendritic cell (so named because of its characteristic elongate cytoplasmic dendrites) is located within or beneath the epithelial barrier where it is well placed to encounter and sample foreign antigen as it is first exposed to the body. The means by which this reaction occurs, and the way in which dendritic cells subsequently direct the nature of the ensuing adaptive immune response, will be discussed in detail below.

Whereas the innate immune system is continually present and able to react immediately to foreign antigen, one major feature of the adaptive response is a delay in onset. This delay is accounted for by the requirement for a sequence of complex intercellular interactions leading to production of antigen-specific T and B effector lymphocytes that must migrate from organized lymphoid tissue (where they are generated) to the site of antigenic exposure via the lymphatic and blood vascular systems. The adaptive immune response also encompasses the generation of populations of antigen-specific regulatory (i.e. suppressor) lymphocytes that control the immune response by down-regulating (suppressing) effector cells when they are no longer required to

be active. Immunological memory is the lasting sequel to this regulatory process.

T CELLS

Definition and Development

T cells are defined by the expression of a T cell receptor (TCR) that confers unique antigen specificity to the cell. The TCR complex comprises a central two chain transmembrane portion involved in antigen recognition that is associated with a series of minor components (collectively called CD3 and comprising γ , δ , ϵ and ζ chains) that act as signal transducers following recognition of specific antigen (Fig. 51.1). The majority of T cells utilise α and β chains in forming the central portion of the TCR complex, and a second group of T cells have $\gamma\delta$ TCRs. The diverse specificity of TCRs is achieved by the variable genetic combination of an array of gene segments that encode portions of the TCR chains (see Chapter 10). The total repertoire of TCRs in the body is sufficient to permit an animal to potentially recognise any possible antigen that it may encounter. Most mature T cells also express one of two mutually exclusive surface molecules, CD4 or CD8 that define non-overlapping T cell subsets; however, double negative (CD4⁻ CD8⁻) or double-positive (CD4⁺ CD8⁺) T cells are found at certain stages of development.

All lymphoid cells, including T cells, arise from the common bone marrow stem cell (see Chapter 10). Immature T cell precursors are then exported from the marrow to undergo development and maturation in the thymus.^{4,37} Intrathymic development of T cells is

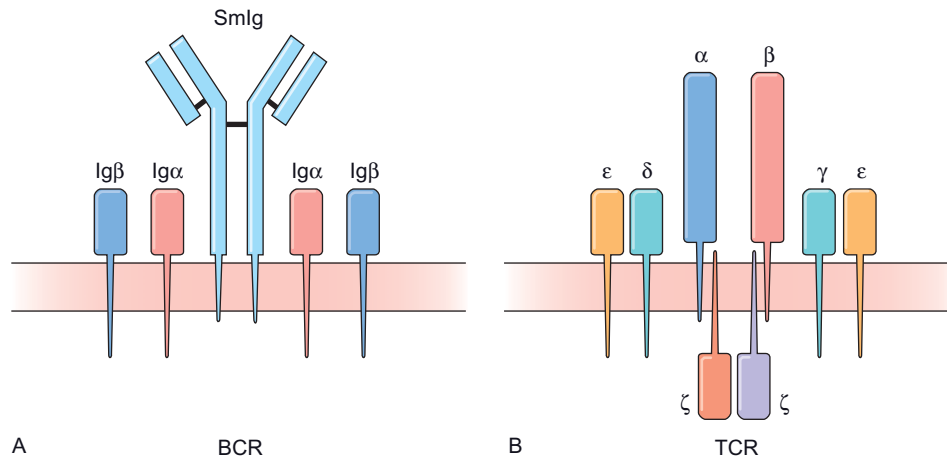


FIGURE 51.1 Diagrammatic representation of the B cell receptor (BCR) and T cell receptor (TCR). Antigen recognition is mediated by the surface membrane immunoglobulin of the BCR, or the $\alpha\beta$ chains (alternative $\gamma\delta$ chains not shown) of the TCR. The signal transduction molecules of the BCR are collectively known as CD79, and those of the TCR as CD3.

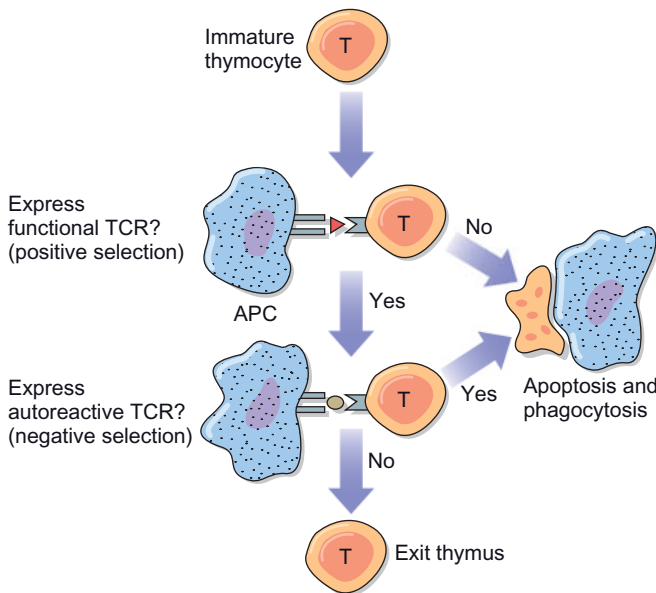


FIGURE 51.2 Intrathymic development of T cells. Developing thymocytes must express a T cell receptor able to productively interact with MHC and peptide, but must not recognize self-antigens that are capable of inducing an autoimmune response. Only T cells that satisfy these requirements are permitted to leave the thymus and enter the peripheral recirculation pathway.

complex, and involves a series of interactions between the developing thymocytes and populations of antigen-presenting cells (APCs) such as thymic epithelial cells or dendritic cells. These tests of developing thymocytes determine whether the cells express a functional TCR capable of antigen recognition (positive selection) or a TCR that recognizes self antigens and induces autoimmunity (negative selection); T cells that fail either test undergo apoptosis (programmed cell death or cell suicide) within the thymus and the remnants of the cell are phagocytosed by macrophages (Fig. 51.2).

Mature CD4+ or CD8+ T cells leave the thymus and seed the peripheral lymphoid tissue, particularly the lymph node paracortex, splenic periarteriolar lymphoid sheath, or perifollicular regions of mucosa-associated lymphoid tissue (Fig. 51.3).

However, lymphocytes do not remain static within these locations. There is continual and massive recirculation of lymphoid cells throughout the body, via the pathway depicted in Figure 51.4. Such recirculation is necessary in order to optimize the chance of contact with specific antigen, and to orchestrate and regulate an immune response in appropriate areas of the body. Lymphocyte recirculation and vascular egress of these cells is carefully regulated by a complex network of adhesion molecules expressed by the recirculating lymphocytes and modified vascular endothelium (high endothelial venules; HEV) found normally in some lymphoid tissues or induced at sites of inflammation.^{1,35}

Lymphocytes are considered functionally naïve until they encounter the antigen that they are programmed to recognize via their specific TCR; following participation in an immune response, a population of memory lymphocytes persists for generation of the more effective anamnestic (memory) immune response.

T Cell Activation

T cells have specialized requirements for activation. Intact antigen is generally unable to stimulate T cells and must first be processed and presented by populations of APCs.⁶ Naïve T cells are most effectively activated when antigen is presented to them by dendritic cells, but macrophages and B cells can present antigen, and in some circumstances a wide variety of other cells (e.g. endothelia, epithelia, fibroblasts) can be induced to present antigen (non-professional APC).

Major breakthroughs have been made in expanding understanding of the key role of dendritic cells in T cell activation. Although considered part of innate immu-

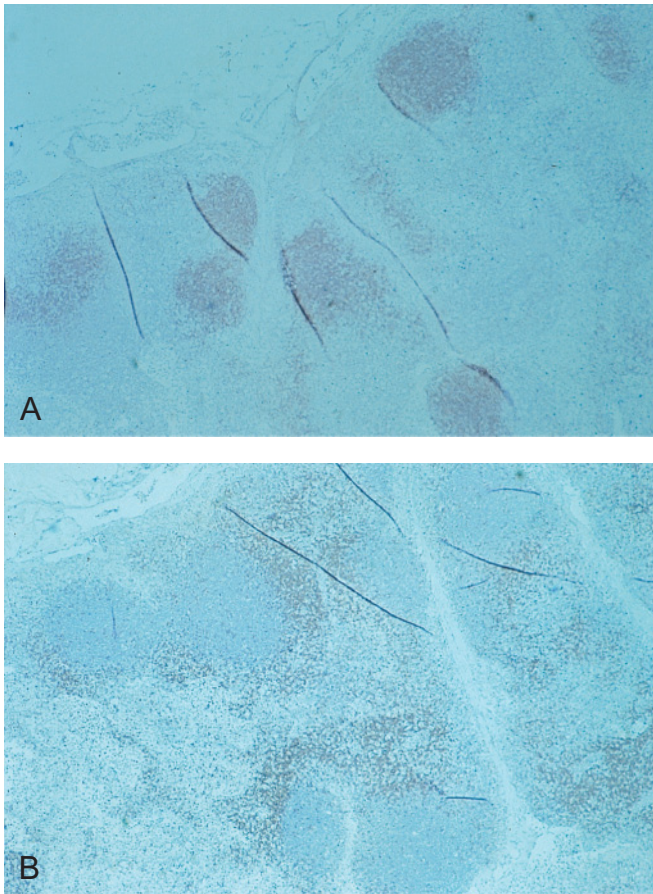


FIGURE 51.3 Sections of canine lymph node immunohistochemically labelled for expression of (A) CD79 and (B) CD3. The two antibodies clearly delineate cortical aggregates of B cells (A) and surrounding paracortical T cells (B).

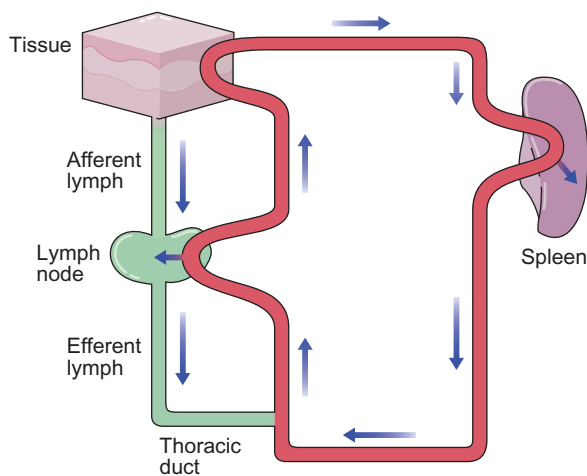


FIGURE 51.4 Pathways of lymphocyte recirculation. Lymphoid cells from the tissues drain to regional lymph nodes via the afferent lymph and exit the lymph node through efferent lymph, subsequently entering the blood via the thoracic duct. Lymphocytes circulate in the bloodstream and may leave the vasculature to enter lymphoid tissue or other body tissues when there is expression of appropriate adhesion molecules by the local vascular endothelium.

nity, it is now known that the dendritic cells direct the nature of the specific adaptive immune response and that this in turn is dependent on the nature of the stimulating antigen. The dendritic cell is endowed with a series of surface and cytoplasmic molecules collectively termed pattern recognition receptors (PRRs). The surface PRRs are also known as Toll-like receptors (TLRs) after the molecule Toll that was identified in the fruit fly *Drosophila* as a key immune-defense molecule in that insect.³³ TLRs are few in number and are highly conserved throughout evolution. Cytoplasmic PRRs include the NOD- and RIG-like receptor molecules.³⁹

The ligands for PRRs are antigenic epitopes expressed by microbes or damaged tissue cells. The former are known as pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs) and the latter as damage-associated molecular patterns (DAMPs). These are structurally conserved molecules that may be protein, carbohydrate, or nucleic acid in origin. Particular PAMPs interact with combinations of specific PRRs; for example, the peptidoglycan of Gram-positive bacteria binds to TLR-2, whereas the lipopolysaccharide of Gram-negative bacteria interacts with TLR-4. The surface PRRs are associated with signal transduction molecules such as MyD88 and ligation of the PRR triggers an intracytoplasmic and intranuclear signaling pathway that leads to differential gene expression via nuclear factor kappa B (NF κ B) and other transcription factors. This gene expression determines how the APC will signal the responding T cell (as discussed below) and therefore determines the nature of the ensuing T cell response.

In parallel with these signalling events, antigen is also internalized by the APC, processed and presented on the cell surface in association with molecules of the major histocompatibility complex (MHC). There are two broad pathways of antigen presentation. Most exogenous antigens (the majority of antigens such as infectious agents or allergens) are taken up by phagocytosis or macropinocytosis and placed in a phagosome where they are enzymatically degraded to small peptide fragments that associate with Class II molecules of the MHC. The combination of antigenic peptide-MHC is then expressed on the surface of the APC in a manner that can be recognised by the TCR. Endogenous antigens, that are derived from the cell cytoplasm, (e.g. viral, tumor or self molecules), undergo an alternative process involving degradation within a cytoplasmic proteasome and transport into the endoplasmic reticulum (mediated by transporter proteins), where they associate with Class I MHC molecules. After transfer to the Golgi apparatus, the combination of antigenic peptide-MHC is similarly expressed on the surface of the APC (Fig. 51.5).

Endogenous peptides also can be presented by MHC Class II molecules and exogenous peptides by MHC Class I molecules in a phenomenon known as cross-presentation. Certain lipid antigens (e.g. derived from *Mycobacterium*) are presented via a third pathway involving endosomal process and presentation in association with molecules of the CD1 family.⁷ T cells that

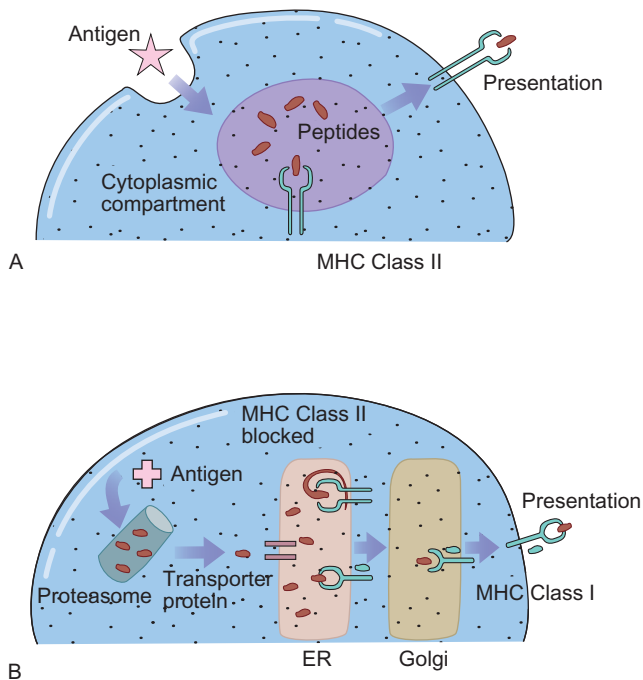


FIGURE 51.5 Processing and presentation of (A) exogenous antigen and (B) endogenous antigen. Exogenous antigen is degraded in a cytoplasmic compartment where there is association between peptide fragments of antigen and class II molecules of the major histocompatibility complex. Endogenously derived antigen is degraded within cytoplasmic proteasomes and transported to the endoplasmic reticulum where peptide fragments become associated with MHC class I molecules. In each case, the processed antigen is presented on the surface of the antigen-presenting cell by MHC for T cell recognition.

recognize lipid antigen expressed by CD1a, CD1b, or CD1c are likely similar in nature to peptide-specific T cells, whereas only NKT cells (see below) recognize antigen expressed by CD1d.¹³

T cell activation is, therefore, a complex event that is driven primarily by the APC. A series of molecular interactions occurs between these two cells that lead to activation of T cells of a specific type that is most relevant to the inciting antigen. These molecular events include: (1) Recognition of the combination of antigenic peptide and MHC residues by the TCR leading to signalling via CD3; (2) cognate interaction of the APC and T cell via an array of other surface molecules (e.g. B7 and CD28). In one such interaction, the CD4 molecule binds to MHC Class II and CD8 to MHC Class I. This renders the CD4+ T cells susceptible to stimulation only when antigen is presented by Class II, and CD8+ T cells to activation only following recognition of peptide in the context of Class I; (3) release of co-stimulatory cytokine/s by the APC that bind cytokine receptors on the T cell. The nature of cytokines released by the APC is determined by the PAMP-PRR interaction. APC-derived co-stimulatory cytokines bind specific cytokine receptors on the surface of the T cell, and when aggregated such receptors signal via activating cytoplasmic tyrosine kinases (Janus kinases) that phosphorylate the

cytosolic signal transducers and activators of transcription (STATs), subsequently migrate to the nucleus, and initiate gene activation via the effects of specific transcription factors.

Following activation, the T cell undergoes blast transformation and cytokine secretion (e.g. interleukin 2; IL-2), and the process of clonal proliferation and differentiation, whereby large numbers of T cells with identical antigen-MHC specificity are generated to participate as effector cells in the immune response. Such T cell activation generally occurs within the secondary lymphoid tissue (e.g. lymph node paracortex) and activated T lymphocytes enter the recirculation pathway to arrive at the site of antigen exposure, or other lymphoid tissues.

T Cell Functions

The end effect of T cell activation is to orchestrate some component of an antigen-specific immune response. T cells may be regulatory, providing either positive (helper) or negative (suppressor) signals to other leukocytes, or be cytotoxic in function.

Function of CD4+ T Cells

T cells bearing the CD4 molecule regulate the function of a variety of leukocytes or cross-regulate the function of each other. The recognition of CD4+ T cell subsets has fundamentally reshaped study of the immune response in recent years.³² First recognized were two subsets of $\alpha\beta$ TCR+ CD4+ T cells, known as Th1 (T helper) and Th2. Although phenotypically indistinguishable, these subpopulations have distinct function conferred upon them by the secretion of two non-overlapping cytokine profiles (Fig. 51.6).

Th1 cells selectively secrete the cytokines IL-2 and interferon gamma (IFN- γ) and enhance the cytotoxic effects of CD8+ and natural killer (NK) cells, the killing of intracytoplasmic pathogens (e.g. *Leishmania*, *Mycobacterium*) by macrophages, and the selective production of antibody of a specific IgG subclass (IgG2a in mice). Th2 cells selectively secrete IL-4, -5, -6, -9, -10, and -13, provide help for B cell and plasma cell development, and promote secretion of IgE, IgA, and another subclass of IgG (IgG1 in mice). The two subsets are mutually exclusive in function as the cytokines produced by each are antagonistic of the other population. For example, IFN- γ will down-regulate the function of Th2 cells, whereas IL-4, -10, and -13 are suppressive of Th1 function either directly or indirectly through influencing the function of the APC. In practice, this exclusivity (known as immune-deviation) is not absolute, as elements of Th1 and Th2 immunology are involved in many immune responses, although some examples of distinctly polarized responses exist (e.g. Th1 requirement for resolution of intracellular infections; Th2 requirement for expression of type I hypersensitivity disease). Th1 and Th2 cells may follow selective recirculation pathways to different tissue sites as they may express unique adhesion molecules,⁵ and be chemotac-

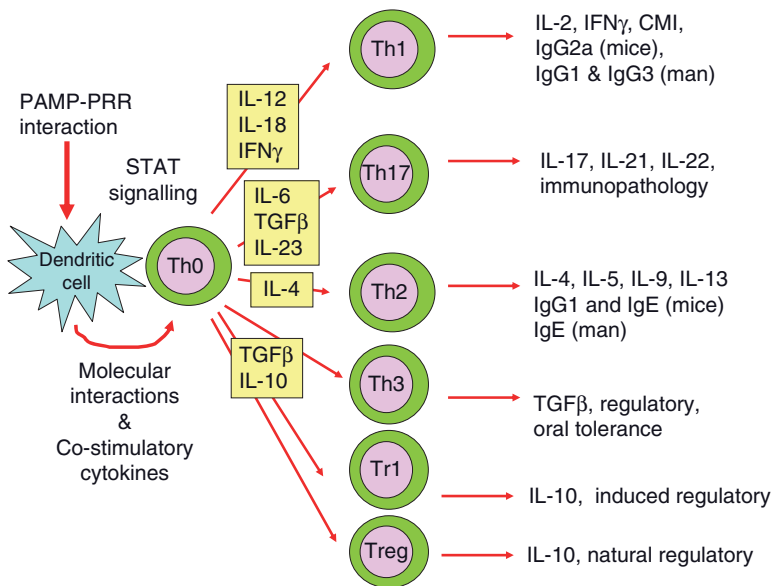


FIGURE 51.6 The functional subsets of CD4⁺ T cells produce distinct cytokine profiles and mediate different immune functions. The subsets have a common precursor (Th0) and a number of factors determine which of the subsets will predominate in any particular immune response, in particular the nature of the stimulating antigen and its interaction with dendritic cell pattern recognition receptors, which in turn determines the type of co-stimulatory cytokine and STAT signalling that stimulates the naïve precursor lymphocyte. Th1 cells regulate cell-mediated immunity whereas Th2 cells control humoral immune responses. Th17 cells have a proinflammatory effector role and are frequently activated in parallel to Th1 cells. The three key regulatory populations are T-reg “natural” suppressors, Tr1 “induced” suppressors, and Th3 regulatory cells.

tically attracted by specific molecules (chemokines) for which they bear receptors.¹¹ Th1 and Th2 cells are now characterized in several domestic animal species and their role in particular diseases has been defined.²²

Th1 and Th2 subsets are proposed to be related through a common Th0 precursor that secretes elements of both cytokine profiles. In any particular immune response, the response can be driven towards either type 1 (Th1) or type 2 (Th2) function by a range of factors including:

- antigen type (e.g. the nature of an infectious agent), dose, and route of delivery
- the nature of the APC presenting the antigen (e.g. dendritic cells or non-professional APC)
- local cytokine and hormonal environment in the lymphoid tissue generating the response; for example, the presence of high concentrations of endogenous corticosteroid or IL-4 may preferentially activate a Th2 response, whereas IL-12 will drive forward a Th1 response
- APC signalling as determined by the PAMP-PRR interaction. For example, viral RNA acting as a PAMP will induce dendritic cell production of IL-12 and IL-18 as co-stimulatory cytokines, resulting in signalling through STAT-4 and regulation through the transcription factor T-bet with induction of a Th1 immune response most appropriate for the viral pathogen. By contrast, antigen derived from a helminth induces IL-4 and IL-6 signalling, activation via STAT-6 and the transcription factor GATA3 with generation of a Th2 response best equipped to counteract the effects of such parasites.

A further CD4⁺ effector T-cell subpopulation has recently been identified. The Th-17 cell is engendered when Th0 precursors are signalled via IL-6, IL-23, and transforming growth factor (TGF)- β . Th-17 cells produce IL-17, IL-21, and IL-22 and have proinflammatory effects that are important in the clearance of certain

classes of pathogen and the pathogenesis of some cell-mediated autoimmune diseases.²⁹

The final CD4⁺ T cell subsets are regulatory (suppressive) rather than effector in nature. The most important of these is the CD4⁺ CD25⁺ natural T regulatory cell (T-reg).^{2,23,30} These cells are spontaneously present and have a key role in controlling potentially deleterious autoimmune and allergic immune responses. They produce IL-10 but require direct physical contact (cognate interaction) with the cell that they are suppressing. T-reg also express Foxp3 (forkhead box P3 gene), GITR (glucocorticoid-induced TNF-receptor-regulated gene), CTLA-4 (cytotoxic T lymphocyte antigen 4), and some PRRs. T-reg have now been characterized in cats, dogs, and cattle.^{10,40} By contrast, the induced regulatory T cells (Tr1 cells) appear as part of an immune response to foreign antigen (as opposed to being present naturally) and function mainly through production of the immunoregulatory cytokine IL-10 rather than requiring direct cognate interaction with their targets.¹⁹ These cells have recently been characterized in the horse.⁴¹ Intriguing recent studies have shown that in the late stages of some infectious diseases (classically leishmaniasis) protective Th1 cells may switch their functional phenotype from an effector IFN- γ production to an IL-10 producing regulatory population. This has the effect of permitting persistence of infection but limiting immune-mediated damage to tissue that may be secondary to an over-exuberant protective immune response.^{5,38} Finally, the Th3 cell preferentially produces TGF- β and has been suggested to mediate the phenomenon of oral tolerance (failure to respond to systemic administration of antigen after prior feeding of the antigen).⁴²

Function of CD8⁺ T Cells

The major function of CD8⁺ T cells is mediating cytotoxicity, and these cells are positively influenced by the

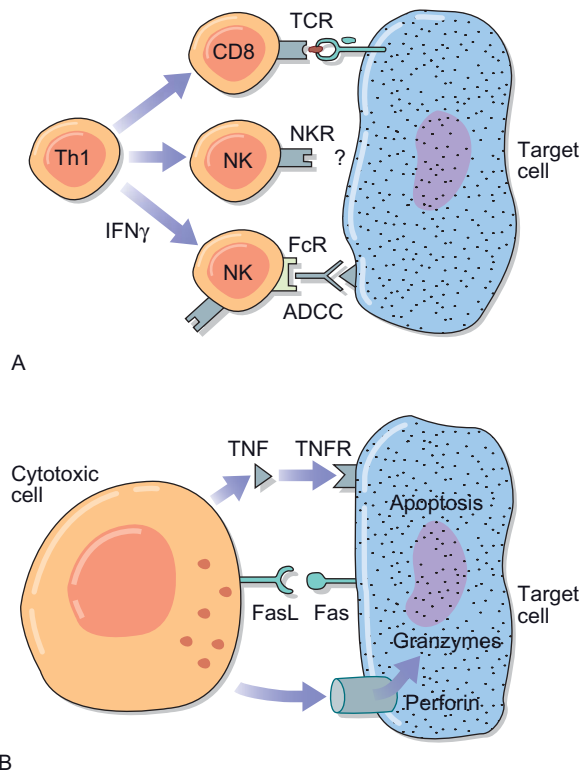


FIGURE 51.7 (A) Cytotoxic destruction of a target cell can be mediated by CD8+ T cells or natural killer cells. These populations are positively influenced by Th1 CD4+ T cells. (B) The cytotoxic process involves induction of target cell apoptosis by the interactions of Fas-Fas ligand and TNF-TNF receptor, membrane pore formation and osmotic imbalance, and cytoplasmic damage caused by granzymes released from the cytotoxic cell.

effects of the Th1 regulatory population. However, it has been recognized that CD8+ T cells are capable of producing type 2 cytokines (e.g. IL-4) and subsets of type 1 and type 2 CD8+ T cells are proposed.¹⁴ The cytotoxic effect of lymphoid cells follows a similar sequence of events (Fig. 51.7) that are initiated following recognition of the target cell (e.g. virally infected, neoplastic, or incompatible graft cell) by cytotoxic cells.¹² In the case of CD8+ T cells, the recognition involves TCR interaction with endogenous peptide presented by MHC Class I. A range of other surface molecular interactions between the two cells is also required. After recognition, the cytotoxic effects are mediated by:

- induction of target cell apoptosis triggered by intracellular signals delivered following molecular interactions between Fas (on the target cell) and Fas-ligand (on the cytotoxic cell), or cytotoxic cell-derived TNF binding to TNF receptor on the target cell
- osmotic and enzymatic effects produced by the release of substances from the cytotoxic cell that form membrane channels in the target cell (perforins) permitting osmotic imbalance, or the delivery of toxic substances (granzymes) to the target cell cytoplasm.

Cytotoxic cells may then disengage from the killed target cell and subsequently attack other targets.

Function of $\gamma\delta$ T Cells

In most species, T cells expressing the $\gamma\delta$ TCR with a CD3 complex are primarily located in the skin and mucosal sites of the body; however, in ruminants a significant proportion of blood T cells may be of this phenotype²¹ and the spleen of cattle and dogs are also enriched for these cells.²⁷ $\gamma\delta$ T cells are poorly characterized but are known to develop in the thymus early in ontogeny. The $\gamma\delta$ TCR has limited heterogeneity and may largely recognize conserved microbial (especially bacterial) molecules and thus have a role in the early immune response to such agents. The receptor may recognize antigen directly, or in association with particular forms of MHC class I molecules. Functional subsets of $\gamma\delta$ T cells able to produce either IFN- γ or IL-4 have been proposed, and may be important in creating a cytokine milieu for subsequent development of the CD4+ $\alpha\beta$ + T-cell response.¹⁵

NKT Cells

NKT cells are a T cell subset distinct from CD4+ and CD8+ T cells that are not yet recognized in domestic animals. Two NKT subsets are reported. Type I NKT cells express a T cell receptor that comprises an invariant α chain combined with a limited number of β chains. Type II NKT cells have greater variability in α chain usage. Both cell types are also defined by recognition of antigen expressed by the CD1d molecule, and therefore predominantly respond to glycolipid molecules.¹⁷

NATURAL KILLER CELLS

The NK cell is observed microscopically as a large granular lymphocyte (LGL) and is defined by the presence of a series of NK stimulatory receptors that mediate the cytotoxic interaction with the target cell (Fig. 51.7). Such receptors are not encoded by genes that undergo recombination in the same way as those that encode T cell and B cell receptors (via the recombination-activating gene; RAG). This feature indicates that NK cells are considered part of the innate immune system. The cytotoxic function of an NK cell can be inhibited by the interaction of a second class of receptor (killer inhibitory receptors; KIRs) which recognize MHC Class I molecules on the target cell.²⁸ The balance between stimulatory and inhibitory receptors determines whether the NK cell will be activated to mediate cytotoxic destruction of the target.³¹ The NK cell also may recognize the target cell via interaction of the membrane Fc immunoglobulin receptor and antibody bound to the target cell in the process of antibody-dependent cell-mediated cytotoxicity (ADCC; Fig. 51.7).

B CELLS AND PLASMA CELLS

B cells are defined by the expression of surface membrane immunoglobulin (SmIg) with a transmembrane

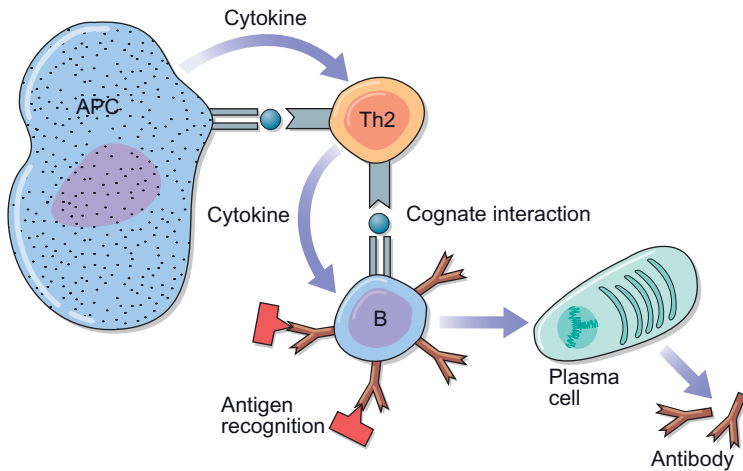


FIGURE 51.8 Activation of B cells requires recognition of antigen by surface membrane immunoglobulin and a range of interactions with helper Th2 cells. These latter include direct interaction of membrane molecules and binding of T cell-derived cytokines by specific receptors. B cell differentiation involves transformation to a plasma cell that secretes immunoglobulin.

domain that is associated with the signal transducing molecules $Ig\alpha$ and $Ig\beta$ (CD79). The complex is collectively referred to as the B-cell receptor (BCR). Each B cell expresses a unique BCR of single specificity and this diversity in the B cell repertoire is achieved by genetic combination of BCR gene segments in a similar fashion to formation of the TCR. A range of other surface molecules (e.g. Fc and complement receptors) are expressed by B cells.

B cells are derived from the bone marrow stem cell and are thought to undergo their development and maturation within the fetal liver and bone marrow of mammals. In some species, evidence suggests that the intestinal tract may be an alternative site of B cell development. The stages of B cell development are less well understood than for the T cell, but the principle of cellular interactions with marrow stroma, with deletion of nonfunctional or autoreactive B cells by apoptosis, is likely similar. B cell development is reliant upon direct contact with stromal cells that also provide appropriate growth factors (e.g. IL-7).²⁹ Developing B cells first express cytoplasmic immunoglobulin μ heavy chain, and following development to the naïve stage, express antigen-specific SmIg of both the IgM and IgD classes. B cells are exported from the marrow to particular anatomical locations (lymphoid follicles of the lymph node cortex, splenic white pulp, or mucosal lamina propria; Fig. 51.3) and recirculate throughout the body in the manner described for T cells.

B cell activation occurs in lymphoid tissue and requires a similar array of signals to those described for the T cells. The SmIg of the BCR recognizes antigenic epitopes directly, without the need for antigen processing or presentation by APC. The epitope may be larger and have conformational or planar shape, although peptides may also be recognized. B cells require costimulatory signals delivered by CD4⁺ helper T cells (Th2 or Th1). These take the form of cognate interactions between surface membrane molecules on the two cells (including TCR recognition of antigenic peptide presented on MHC Class II by the B cell) and cytokines derived from the T cell that bind receptors on the B cell

(Fig. 51.8). Some antigens (thymus-independent antigens) are able to directly activate B cells in the absence of T cell help.

After activation, the B cell undergoes transformation to a lymphoblast (that may secrete IgM) and clonal proliferation and differentiation as described for T cells. As part of the differentiation process, the B cell undergoes the immunoglobulin class-switch, involving DNA rearrangement with substitution of the μ and δ genes by one of the other constant region genes to produce SmIg of the IgG, IgA or IgE class. This process appears to be cytokine-directed.

The initial contact of antigen-specific B and Th2 cells with APC (dendritic cells) and antigen occurs in the T cell zones of lymphoid tissue (e.g. lymph node paracortex) following egress of the T and B cells from the HEV. At this point, there is B cell proliferation and activated B and T cells migrate from this primary focus to the B cell area (primary follicle) to form a germinal centre within the follicle.²⁶ These activated B cells (centroblasts) accumulate within the dark zone of the germinal center of this secondary follicle, and then migrate to the edge of the germinal center (light zone) where as centrocytes they re-encounter antigen on follicular dendritic cells. The mantle zone of this secondary follicle is composed of inactive B cells that are not specific for the antigen driving the immune response. Most of the centrocytes have BCRs that recognise antigen with low affinity and this interaction causes them to undergo apoptosis; however, those centrocytes with appropriate receptors undergo further interactions with Th cells, and leave the germinal centers to differentiate into plasma cells (that secrete immunoglobulin but do not have SmIg), or the population of memory B cells that mediate the secondary immune response on re-encounter with antigen.²⁴ Plasma cells migrate as precursor plasmablasts and are largely located in lymph node medullary cords, splenic red pulp, bone marrow and the mucosal lamina propria. They have a limited lifespan (weeks) at these sites. Memory T and B cells are long-lived cells that may be periodically restimulated by depots of antigen that persist associated with

dendritic cells in lymphoid tissue, or are reintroduced to the body by vaccination or exposure to microorganisms bearing cross-reactive epitopes.^{8,18}

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Structure, Function, and Disorders of Lymphoid Tissue

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Hemopoietic System Cells and Organs
Functional Anatomy of the Hemopoietic System
Thymus

Spleen
Lymph Nodes
Lymphoid Systems of Body Surfaces

Acronyms and Abbreviations

BLV, bovine leukemia virus; FeLV, feline leukemia virus; MALT, mucosa-associated lymphoid tissue; MHC, major histocompatibility complex; TCR, T cell receptor genes; RBC, red blood cell.

HEMOPOIETIC SYSTEM CELLS AND ORGANS

The hematopoietic system consists of the cascade of cells produced by the bone marrow as well as their specialized conducting and supporting systems consisting of vascular endothelium and the connective tissue cells of the marrow, lymph nodes and spleen.^{5,15,20,21,25}

Other highly specialized supporting structures of the hematopoietic system include the epithelial cells of the thymus which sheath the blood vessels and form the sacular structures of the thymic cortex by which developing T cells gain their recognition of normal self antigens. The hematopoietic cells consist of the full range of differentiated products of pluripotential stem cells, including monocyte-macrophage and granulocytic cells of the neutrophil, eosinophil, and basophil type as well as the precursors of red blood cells (RBCs), platelets, and lymphocytes. The latter, including the thymic and bone marrow dependent arms of the lymphoid system are respectively responsible for cellular and humoral immunity.¹⁵ The vascular endothelium of the hematopoietic system includes the apparently regionally undifferentiated cells lining the lymphatics as well as the regionally differentiated endothelial cells of the blood vascular system which include the high endothelial venules of the lymph node paracortex with specific cell surface markers permitting the adhesion of lymphocytes in transit and their transmural migration to enter the node paracortex.¹⁴ The specialized circulating blood cells and their conducting and supporting structures are uniquely packaged in a series of organs of either separate design like the thymus, lymph nodes,

and spleen or are incorporated into other organs like the bone marrow. These also include the free and fixed tissue macrophages and dendritic cells. These cells and organs of the blood vascular system constitute remarkably interrelated and integrated systems

FUNCTIONAL ANATOMY OF THE HEMOPOIETIC SYSTEM

Thymus

The thymus is cytologically simple but unique in containing both lymphocytes and epithelial cells, but is architecturally complex in having a lobular structure differentiated into cortical and medullary areas (Figs. 52.1 and 52.2). The epithelial cells of the thymus are derived from the third and fourth pharyngeal pouches that, in the embryo, migrate in two streams to form paired lobes of the thymus within the anterior mediastinum. This migration occurs very early in embryological development and is immediately followed by seeding of the thymus with progenitor cells from the blood islands of the yolk sac.¹⁵

The first stream of epithelial migration forms the isolated reticular epithelial cells of the cortex and medulla of the adult thymic lobule. The second epithelial migration forms the thymic duct epithelium and later the Hassall's corpuscles of the thymic medulla (Fig. 52.3).²¹ Early reticular epithelial cells form loose cuffs around small vessels that persist in adult life and become obvious in conditions of lymphoid atrophy.

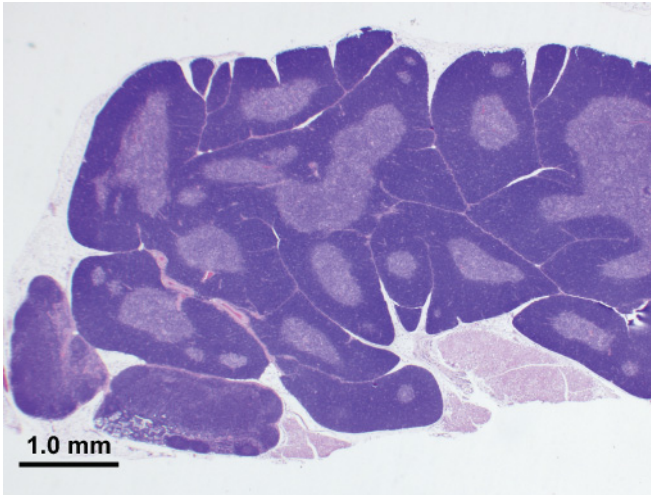


FIGURE 52.1 Thymus from a young adult male rat. The organ is composed of closely faceted lobules with a sharp distinction between the darker cortex and the lighter medullary areas of each lobule. Hematoxylin & eosin stain; bar = 1.0 mm.

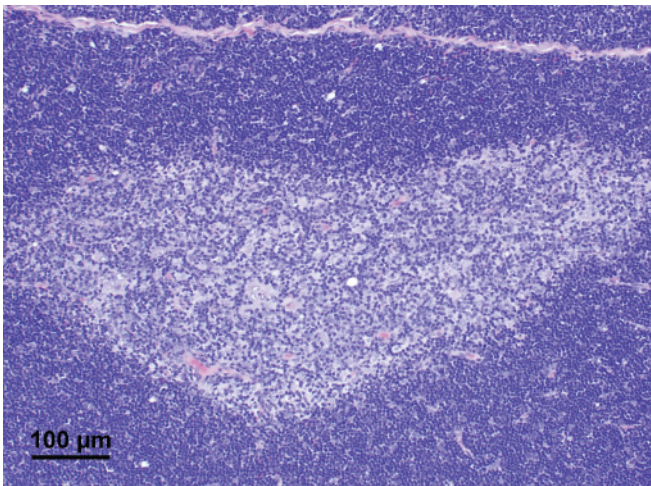


FIGURE 52.2 Mammalian thymus. In mammals, the medulla and cortex is sharply delineated in young healthy animals with the width of the cortices and medulla approximately equal to one-third the width of the lobe. Hematoxylin & eosin stain; bar = 100 μm.

Also, in early development, the ductular component of epithelium forms the branching system that communicates between the lobules of a single thymic lobe. These embryological relationships are important due to the mimicry of embryological events in thymic lesions of adult life. Thus, a thymic lesion with loss of corticomedullary distinction that might be medullary hyperplasia or thymoma can be differentiated by the presence of the reticular cuffs around the vessels seen in a thymoma. These epithelial cuffs of thymic vessels form a barrier to blood-borne antigens and naïve bone marrow lymphocytes receive their immunologic training solely from the reticular epithelial cells. Lymphoid germinal centers occurring in the thymus are not necessarily pathologic or an indication of autoimmune disease if

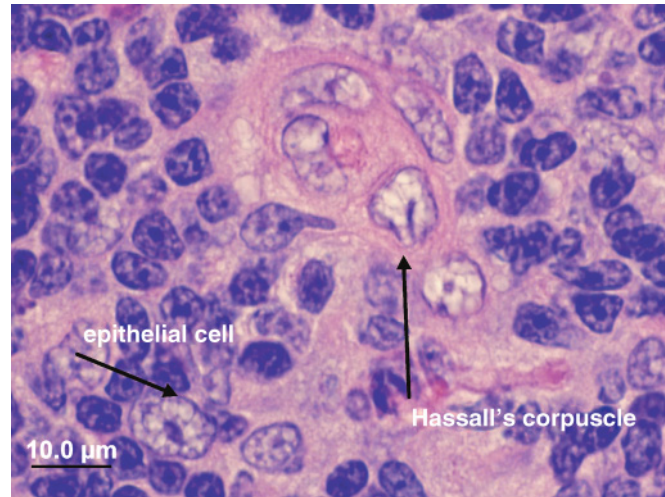


FIGURE 52.3 Hassall's corpuscle in the thymic medullary area consisting of concentric laminations of squamous epithelial cells. The surrounding lymphocytes are characteristically a mixture of small and medium cells and epithelial cells with large pale nuclei (arrow). These cells with moderately abundant cytoplasm separating the nuclei give the medulla a less dense appearance than the cortex on histological examination. Hematoxylin & eosin stain; bar = 10 μm.

they occur near a blood vessel and lie within the vascular epithelial sheath and are thus immunologically outside of the thymus. Detection of this relationship requires application of a cytokeratin stain. The medullary epithelium produces trophic hormones that assist lymphocytic colonization and are the source of cysts lined by ciliated epithelium that frequently develop in adult life but are rarely of clinical significance. An additional cellular component are myoid cells. They surround the Hassall's corpuscles and are important in the pathogenesis of myasthenia gravis.²¹ In most species, the thymus reaches its maximal development about the time of puberty and then slowly decreases in size throughout adult life. An unusual antigenic stimulation during adolescent life may result in benign thymic hyperplasia, which, in the calf, may result in a chain of thymic lobules that extend from the rami of the mandibles to the base of the heart.

At the physiological level, the thymic cortex receives a continuous stream of uncommitted lymphocytes of bone marrow origin which undergo immunological selection for tolerance to self-antigens in contact with the reticular epithelial cells which form thin-walled pouches (caveolae) in cortical tissue (see Chapter 51). Paradoxically, the thymic cortical lymphocytes have small densely stained nuclei without apparent nucleoli, yet this is a region of intense cellular proliferation in which the great majority of the progeny die and are removed by tingible body macrophages (Fig. 52.4). These latter cells become more prominent in conditions resulting in cortical lympholysis such as certain viral infections, irradiation, or corticosteroid therapy. Marrow-derived lymphocytes have the cell surface molecules to selectively home to thymic cortical

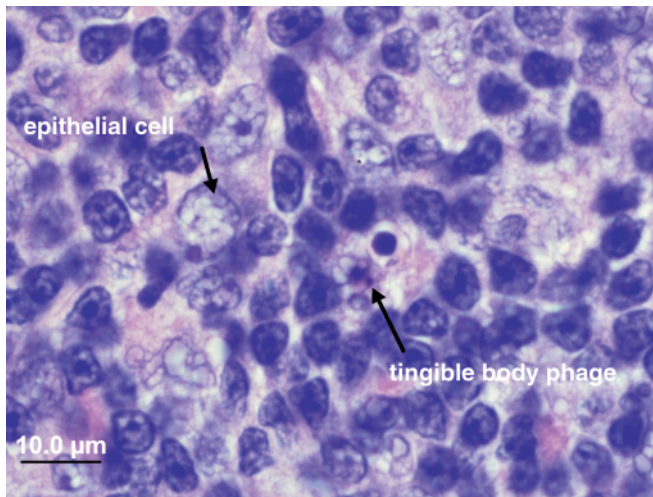


FIGURE 52.4 Thymic cortex composed of densely packed small lymphocytes. A macrophage in the center has cytoplasm that contains several pyknotic nuclear fragments (tingible bodies) of apoptotic cortical thymocytes. The larger vesicular nuclei above and to the left of the macrophage are probably epithelial cells involved in the self-recognition selection process of developing T cells. Hematoxylin & eosin stain; bar = 10 μm.

vascular endothelium. The naïve T cells rearrange their T cell receptor genes (TCR) most to $\alpha\beta$ and some to $\gamma\delta$ type (see Chapter 51).⁵

Medullary lymphocytes are larger than the cortical cells, having larger and more vesicular nuclei, more cytoplasm, and reduced cell density. These cells give the medullary area less density on histological examination when routine stains are applied. Physiologic development continues when lymphocytes enter the medullary areas with exposure resulting in preferential homing of cells to the intestinal mucosa and Peyer's patches.

In young animals, particularly rabbits, heterophils are frequently found in the thymus, particularly in lobular connective tissue. Eosinophils may occasionally be found in the thymic connective tissues of other species and mast cells are present in the thymic capsules in most species and are particularly frequent in rats. Thymic hyperplasia occurs largely through an increase in number of lobules rather than increased lobule size, while thymic atrophy tends to result in a blurring of the corticomedullary distinction. Thymic function does not necessarily vary in proportion to thymic size and in the adult a small thymic remnant may be responsible for a persistent autoimmune disease. Recent work suggests that terminal maturation of T cells may occur in the intestinal tract as well as in the thymus.¹³ It would not be surprising if similar activity was found in both the lung and skin which would provide a more comprehensive explanation for aberrant lymphoid reactions in both benign and malignant states.

Spleen

The spleen filters blood through a sinusoidal system. It has additional functions in lymphopoiesis and antibody

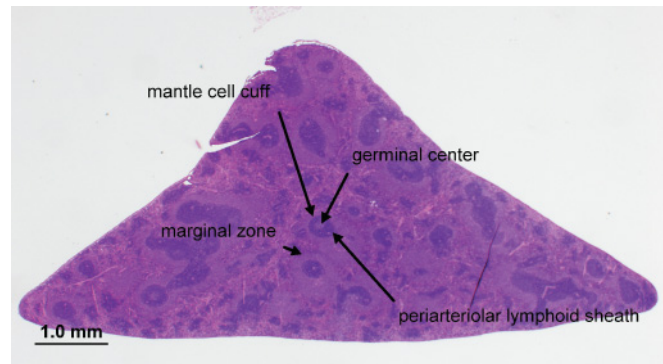


FIGURE 52.5 Cross-section of spleen from a young adult male rat. At the architectural level, the spleen is occupied by multiple, dispersed round dark areas representing lymphoid nodules with the intervening lighter areas consisting of sinuses. The lymphoid or white pulp areas may have a germinal center in the middle of the mantle cell cuff (arrows). These areas are surrounded by the lighter mantle and marginal zone areas. Hematoxylin & eosin stain; bar = 1.0 mm.

production and hemopoiesis under conditions of increased demand for blood cells by colonization of sinusoids with pluripotential stem cells. Species with the genetic potential for sustained activity such as humans, dogs, cats, and horses have spleens with a contractile muscular capsule supported by equally contractile internal trabeculae. In contrast, species such as ruminants, which tend to group together for protection against their more agile predators tend to have spleens whose capsules are largely connective tissue with less capability for contraction. The major hazard to life after splenectomy appears to be septicemia. The spleen forms early in embryonic life and is a site of active erythropoiesis during the fetal period. The spleen is both architecturally and cytologically complex. It contains a wide variety of cells that vary in proportion in reactive and disease states. The spleen also has a diverse regional anatomy based on a complex vascular system able to alter internal anatomy with changes in overall size and volume.

The spleen is unique in having efferent but no afferent lymphatics. Thus, all antigen enters the spleen through the blood vascular system. Both the major arterial supply and venous outflow enter through the hilus of the spleen and arborize together throughout its length. This arborization is relatively random in mammals but in reptiles there is a system somewhat analogous to the bone marrow with a major central venous sinus. At the level of small and medium-sized arterioles, the vessels are sheathed in a cuff of small thymic-derived lymphocytes known as the periarteriolar lymphoid sheaths (Fig. 52.5). Very small branches from these "central" arteries give rise to germinal centers that are foci of B cell proliferation. Surrounding the dense cuff of lymphocytes of the periarteriolar lymphoid sheath is a more loosely aggregated area of lymphocytes of the mantle cell layer that is a mixture of T and B cells. This mantle cell layer surrounds the

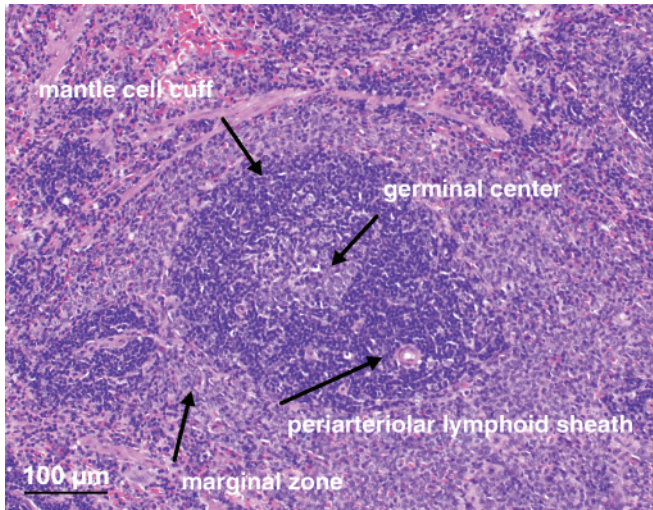


FIGURE 52.6 Details of Figure 52.5. Unlike larger mammals, rats tend not to have well-formed splenic germinal centers. In the middle of the image, a lighter zone constitutes the dendritic cell bed of an ill-defined germinal center eccentric to and abutting a small muscular arteriole at lower right (arrows). The germinal center is surrounded by a cuff of darker mantle lymphocytes that has an outer envelope of lighter-staining marginal zone cells that interface with the surrounding splenic red pulp. Hematoxylin & eosin stain; bar = 100 μm.

germinal centers that arise adjacent to the central arterioles. A marginal zone of B cells lies outside the mantle cells (Fig. 52.6). Whereas the cells of the periaarteriolar sheath and germinal center are composed of resident cells with movement largely through negative selection and replenishment, the lymphocytes of the mantle cell layer interchange with cells of the germinal center. The width of marginal zone cells varies widely with immune activity and is itself surrounded by a trough or sinus area that receives blood coming through the germinal center. This area may appear more red in histological section and contains cells currently in the general circulation; it may be viewed to assess the levels of cells in circulation. In humans and the cat this peripheral sinus or perifollicular zone lies between the mantle cell cuff and the marginal zone layer. The rat and mouse have a definite stromal appearing boundary around the mantle cells and germinal center that is not present in humans or domestic animals. The small branches of the central arteriole may terminate in germinal centers or pass through mantle and marginal zones to terminate in a penicillary array of small branches that feed directly into the sinusoids. The penicillary vessels are ensheathed by a few plump reticular cells forming a contractile ellipsoid which under neural and hormonal control adjusts the level of blood entering venous sinuses.

The venous sinuses of the spleen constitute the expansile regions of the organ and thus may vary greatly in their content of blood in species with a high level of smooth muscle in the splenic capsule. The walls of the splenic sinusoids are unique in consisting of elongated endothelial cells arranged like the staves of a barrel which are without junctions to their neighbors

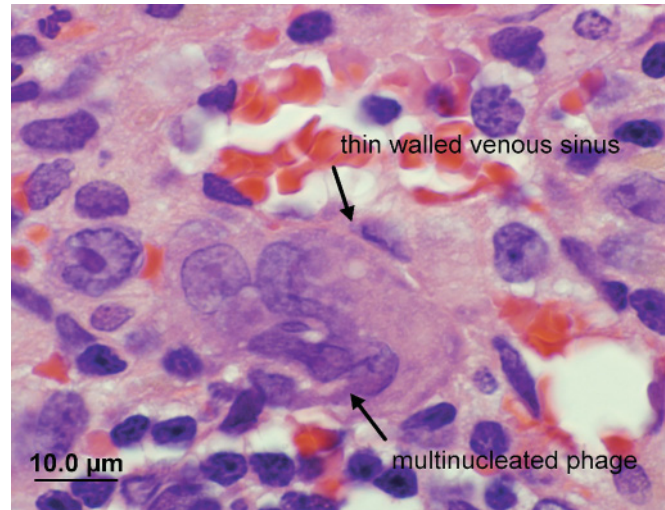


FIGURE 52.7 Splenic sinus area demonstrating the interposition of a large multinucleated macrophage and a thin walled sinus facilitating the filtering system of the spleen. Hematoxylin & eosin stain; bar = 10 μm.

but are maintained in alignment by a discontinuous encirclement of fine reticular fibers (Fig. 52.7). The lumen of these sinuses communicates directly to the exiting vein of the spleen while the exterior of these veins is in contact with the filtering area of spleen that consists of macrophages suspended in a loose reticular supporting framework. Under normal circumstances 97% of the arteriolar blood entering the spleen exists directly via the penicillary vessels into a major sinus and quickly re-enters the central circulation. The remaining 3% passes into the filtering extrasinusoidal area to regain the central circulation by passing between the walls of the sinus endothelial cells. Reticulocytes and normal RBCs and leukocytes readily achieve this transit back into the circulation while senescent RBCs, those containing Howell-Jolly bodies, senescent leukocytes and foreign material, are phagocytosed by the macrophages of the red pulp areas. By this process, the entire blood volume passes through the filtering system of the spleen once each day. In a muscular spleen in the contracted state, all of the blood would pass directly from the arteriole to the venous system in a “closed” circulation, permitting maximal utilization of blood cells in the systemic circulation. In contrast, any influence that causes the splenic capsule to dilate (e.g. anesthesia, portal hypertension due to hepatic fibrosis or marked increase in extrasinusoidal macrophages) results in a greater proportion of blood passing through the filtering system. Due to the slow flow of blood through the red pulp, cells are exposed to lower glucose, cholesterol, and pH than in the central circulation. Collectively, these biochemical changes can contribute to premature aging of RBC and platelets resulting in accelerated destruction. Thus, an enlarged spleen of any cause is a distinct hazard to RBC and platelet lifespan.¹

A variety of changes occur in the spleen in response to systemic states. Acute toxic diseases will result in

lysis of lymphocytes in the germinal centers and replacement by residual proteinaceous debris referred to as follicular hyalinosis. Diagnosticians are aided by the fact that nuclear debris after acute lympholysis is cleared within 24 hours but the hyalin debris may remain for many months. In systemic amyloidosis, the germinal centers may become sites of amyloid deposition, while atrophic changes due to starvation, aging, cancer, or chemotherapy may result in atrophy which impacts one or both of the thymic-dependent arteriolar sheaths or bone marrow-dependent germinal center systems. In chronic hemolytic anemias, a marked increase in hemosiderin-bearing macrophages in sinus areas and iron deposition can occur in the connective tissue of the spleen, particularly in old dogs. Splenic infarction occurs in focal areas of the spleen in dogs with hemangiosarcoma and is always of hemorrhagic type. Ischemic infarction can occur in dogs with myeloma due in part to the greater viscosity of blood. Splenic torsion occurs primarily in dogs and humans and rarely in adult pigs and is surprisingly compatible with life. These events in the dog are apparently painful and dogs with focal infarction due to hemangiosarcoma resist activity and become anorexic even without abdominal hemorrhage.

Lymph Nodes

Histogenetically, lymph nodes form at the confluence of an afferent lymphatic sprig with a dilated sheath of vascular serosa. The blood vessels covered by the serosal sheath then produce a fine arborization of vessels within this area of dilation, thus forming an architectural framework for lymphocytic colonization. The vascular distribution to lymph nodes is highly organized with the arteriolar and venous branches arborizing through the medullary cords to form microcirculatory units that become the functional basis for germinal center formation. Specialized high endothelial venules form in the paracortical areas between, and never within, the germinal centers and present specific adhesion molecules by which transmurial lymphocyte traffic from blood to node paracortex is regulated.³ The normal development of lymph nodes is dependent upon cells and antigens entering through the afferent lymphatics that drain into the peripheral capsule of the node. Virgin (non-activated) lymphocytes apparently circulate randomly in the blood; however, once these cells are involved in antigen recognition in the node cortex, their further migration becomes altered. Activated B cells migrate preferentially to mucosa-associated lymphoid tissues (MALT), while T cells migrate to peripheral lymph nodes.^{5,7} This system of organ-specific lymphocyte homing receptors functions in neoplastic as well as inflammatory conditions, with the spread of malignant lymphocytes limited by their capacity to migrate into various tissues. Malignant lymphocytes tend to mimic their benign counterparts with B cell neoplasms like reactive B cells binding to endothelial venules in certain anatomic sites, including tonsil and lymph node. Thus, the spread of lymphoma to

lymphoid structures is not a function of the tumor cells being able to enter the peripheral blood, but rather an indication that the cells have successfully bound to endothelial venules in order to migrate into additional lymphoid structures.¹⁵ By this understanding, lymphoid neoplasms circulate in the blood, but their degree of dissemination is limited to their binding capability; this has led to the new assessment of leukemia versus lymphoma based simply on the tissue with the greatest volume of tumor.

The afferent lymphatics and the peripheral capsule and the subcapsular sinus are the delivery systems whereby lymph, blood cells, and antigens are delivered to the interior of the lymph node (Fig. 52.8). The peripheral capsule will be thin and taut in conditions in which there has been rapid enlargement of a lymph node of benign or malignant cause. In contrast, lymph node atrophy results in the histologic appearance of a capsule that is thickened and wavy, with the peripheral sinus widened. Chronic stimulation of a node from either hyperimmune or septic cause results in thickening and sclerosis of the capsule with thickening of the fibrous raphe which spans the cortex and terminate in a dense collagenous medullary fibrovascular network.¹⁹ In cattle and likely in other species, the lymph node capsule is contractile and under neural control, thus assisting lymph flow.¹²

The lymph node cortex and germinal centers form the first order of filtering system through channels extending from the inner lining of the peripheral sinus to the microcirculatory units in the node cortex. A fully developed germinal center is a highly organized structure consisting of a progression of cell types in a proliferative gradient that provides the germinal center with an easily recognized polarity (Figs. 52.9 and 52.10).¹⁶

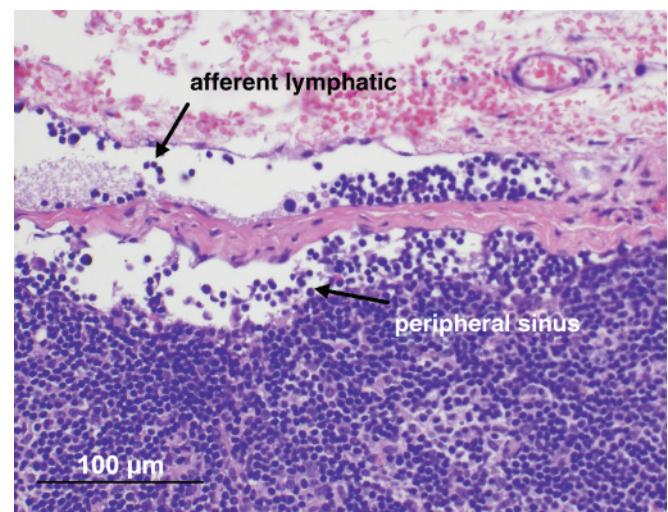


FIGURE 52.8 Lymph node outer cortex from a healthy dog. An afferent lymphatic (arrow) leads into the peripheral sinus beneath the node capsule. The sinus is open and contains a few cells in transit, whereas to the left and right, the sinus is compressed by developing germinal centers. Hematoxylin & eosin stain; bar = 100 μ m.

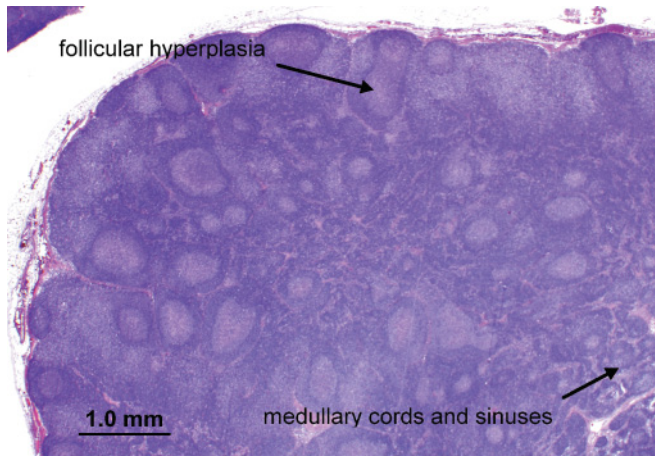


FIGURE 52.9 Architecture of the lymph node in Figure 52.8 showing follicular hyperplasia and densely cellular medullary cords and sinuses (arrows). Hematoxylin & eosin stain; bar = 1.0 mm.

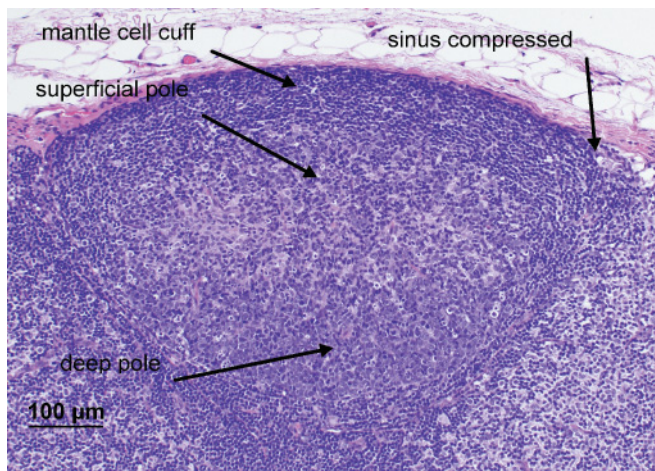


FIGURE 52.10 Lymph node cortex of Figure 52.9 with subcapsular germinal center having a polarity directed toward the antigens being delivered from the peripheral sinus into the superficial pole of small lymphocytes. In the middle of the germinal center, there are small and large lymphocytes with macrophages removing apoptotic lymphocyte nuclei. Near the bottom of the picture, the deep pole area consists of lymphocytes with large nuclei and a narrow rim of highly basophilic cytoplasm. Hematoxylin & eosin stain; bar = 100 µm.

The polarity gradient of the germinal center consists of a mantle cuff of small lymphocytes at the superficial pole, just beneath the inner lining of the peripheral sinus. Antigen sorting and processing occurs through a focusing process, with the antigen presenting dendritic reticular cells in the middle of the germinal center accompanied by tingible body macrophages. Morphologically, the lymphocytes of the superficial pole have small dense nuclei with abundant lightly stained cytoplasm, while those in the deep pole have large nuclei with multiple prominent usually peripheral nucleoli and only a narrow rim of very basophilic cytoplasm. The mantle cell cuff is eccentrically thickened at the superficial or capsular pole and thinned at

the base of the deep pole. In follicular hyperplasias, the germinal centers beneath the node capsule are usually sectioned in a plane that allows their antigen orientation to be recognized, but those in medullary areas are oriented in a variety of planes such that all levels of the proliferative progression of cells will be viewed.⁸ This point is worth emphasizing since it is essential to recognize polarity of germinal centers to distinguish benign and atypical follicular hyperplasias from follicular lymphomas. Follicular lymphomas whether of small cleaved cells of centrocytes, or large cells of centroblasts, or more often a mixture of these, completely lack a mantle cell cuff and are surrounded by a few residual small benign lymphocytes and the aggregations of post-capillary venules. A further distinction of follicular lymphomas is that whatever combination of cell types are present, each follicle will have only that same clonal assembly and lack the variety of benign follicles. Also, unlike benign follicles, those of follicular lymphoma have few or no tingible body macrophages as the tumor cells, unlike the benign cell selection process, are not undergoing apoptosis. A further distinction between true follicular lesions, whether benign or malignant, and pseudofollicular lesions is that the post-capillary venules are always between the follicular or nodular proliferations and never within them.

The dendritic reticulum are derived from a variety of sources and can derive from either myeloid or lymphoid precursors.^{4,9,10,17} Dendritic cells at various anatomic sites have different phenotypic characteristics.¹⁰ Ultrastructurally, the dendritic cells are characterized by cytoplasmic tendrils that are joined by tight junctions. In conditions of lymphoid depletion, the dendritic cells become exposed in germinal centers as the eosinophilic background of large pale cells.

One of the more difficult decisions in examining lymph nodes is the distinction between florid hyperplasia and lymphoma.²⁴ The syndrome of transformation of germinal centers to malignant disease is described in humans^{6,14} and it would appear that in animals chronic lymphoid proliferation of any cause can be a serious risk factor for lymphoma. Examples of this type of interaction in animals include infection with FeLV or BLV and immunoproliferative small intestinal disease of idiopathic cause. A type of nodular or follicular lymphoma in which the malignant cells are derived from the corona of cells surrounding the germinal center occurs in both humans and animals and is known as mantle zone lymphoma. Morphologically, the disease is characterized by coalescing nodular proliferations of moderate-sized lymphocytes surrounding fading germinal centers.

In the paracortex of lymph nodes, there are nodular structures distinct from germinal centers,²⁴ that are particularly common in the mesenteric nodes of mature rats.³ These deep cortical units form as a result of antigenic stimulation, with cellular input from a distinct afferent lymphatic radical (Fig. 52.11). They form semi-circular or oval structures beneath the germinal centers of the outer cortex and are most easily seen in lymph nodes with some degree of paracortical atrophy, making the nodules more apparent. Central areas of these

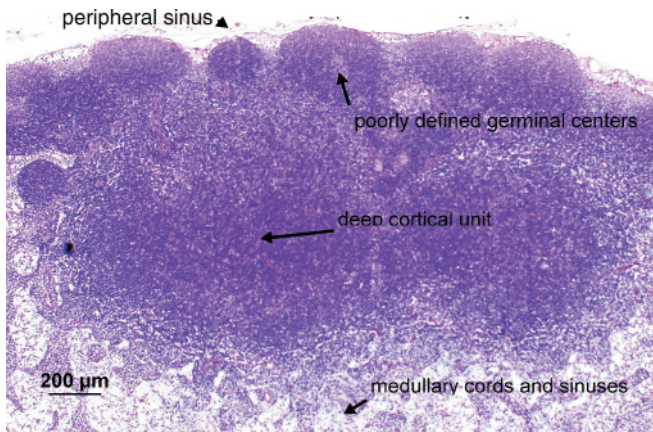


FIGURE 52.11 Cross-section of rat mesenteric lymph node. The upper area of the node is occupied by typically poorly defined germinal centers. The medullary cords and sinuses lie along the bottom of the image and abut a large uniformly dense oval deep cortical unit that lies across the cut surface of the node. The unit is lightly outlined by a rim of less dense cellularity. Hematoxylin & eosin stain; bar = 200 μ m.

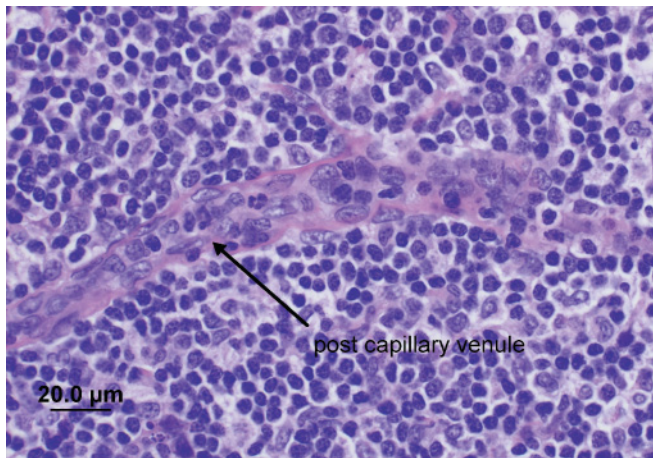


FIGURE 52.12 Paracortex of rat mesenteric lymph node with a high endothelial post-capillary venule crossing the center. Several lymphocytes within the wall of the venule can be seen to be in transmurial transit exiting the blood to enter the node cortex (near arrow). Hematoxylin & eosin stain; bar = 20 μ m.

nodules are somewhat mottled in appearance due to varying proportions of large and small lymphocytes with some macrophages and post-capillary venules (Fig. 52.12). Edges of these nodules gradually meld with the surrounding medullary cords and sinuses and are apparently the areas where cells exiting these nodules enter the efferent lymph channels.³ The main significance of the deep cortical nodules is to recognize that they are normal structures and not to confuse them with a malignant lymphoid proliferation.³

The medullary cords and medullary sinuses are best considered together because they form an interwoven matrix that waxes and wanes in unison or at the expense of each other (Fig. 52.13). A medullary unit consists of a radical of vascular structures from the deep pole of

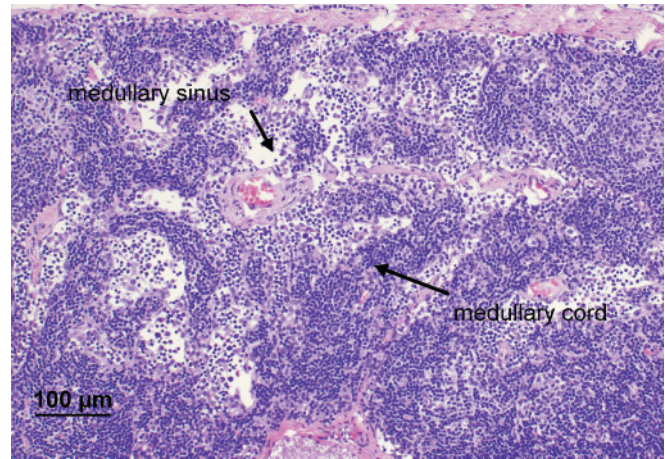


FIGURE 52.13 Medulla of a lymph node from a dog with normal cords and sinuses. The cords are densely cellular and surrounded by a thin endothelial membrane with the sinuses containing a few fixed macrophages and cells in transit in a background of lymph. Hematoxylin & eosin stain; bar = 100 μ m.

the node hilus consisting of an artery, vein, and lymphatic surrounded by a variable amount of connective tissue and invested with a thin endothelial covering. These vascular structures form the center of many medullary sinuses as they are seen in a cross-sectional profile. The sinus area constitutes the space between the endothelial wall of the vascular structures and the similar endothelial wall of the medullary cords. The medullary sinuses may extend up to the inner surface of the subcapsular sinus in nodes in which there is severe paracortical and follicular atrophy. Such a circumstance occurs in the mesenteric nodes of cattle dying of acute bovine viral diarrhea virus infection with massive B cell lysis in which the sinuses tend to be filled with macrophages, thus forming sinus histiocytosis.

The cellularity of the medullary sinuses will vary widely. In diseases characterized by chronic immune stimulation, there usually are large numbers of sinus macrophages which are apparently suspended in a syncytial network. In nodes draining areas of chronic dermatitis, the macrophages of the superficial cortex and those of the medullary sinuses frequently contain abundant normal-appearing melanin granules.¹⁸ Similarly, nodes draining areas of hemorrhage have numerous hemosiderin-bearing macrophages in medullary areas. The transit of RBCs to the lymph node and the process of RBC intracellular degradation is remarkably rapid; thus any amount of erythrophagocytosis by medullary macrophages must be looked on as a very recent event.

The medullary cords contain recirculating lymphocytes that have received antigenic orientation by passage through the outer cortex. The medullary cords may contain small or medium lymphocytes rather than plasma cells, even in reactions that have persisted for many weeks. The fine reticular network of the medullary cords forms a fertile microenvironment for extramedullary hematopoiesis. In myeloid leukemias, the malignant cells also colonize the medullary cords,

and under these circumstances, there may be both tumor and benign extramedullary hematopoiesis coexisting.

LYMPHOID SYSTEMS OF BODY SURFACES

In the context referred to herein, body surfaces include the skin as well as the lining of the upper and lower respiratory and enteric systems. Intraepithelial lymphocytes in all these locations are not primarily cells on the way to being lost to the exterior but rather cells in training, which, following their specific sensitization, return to deeper lymphoid structures to perform a specific function in differentiation and proliferation.

In the skin, keratinocytes constitute 85% of the epidermis with the remaining cells constituting melanocytes, Langerhans' cells, Merkel cells and dendritic cells of uncertain type. In humans and cattle, the Langerhans' cells contain a typical cytoplasmic organelle visible on ultrastructure that is rod-shaped with a unipolar bulb, the Birbeck granule. The Langerhans' cells are products of a variety of precursors and constitute the major antigen presenting cell of the epidermis.¹ The presence of Birbeck granules permitted identification of Langerhans' cells in the paracortex of lymph nodes draining skin. These cells ingest foreign molecules without degrading them and migrate to the local lymph nodes where these antigens are exposed to naïve lymphocytes in specific MHC context. Both T and B cells are present in the skin where their function is largely unknown.² A fairly consistent proportion of cutaneous lymphocytes are suppressor T cells which may be resident in situ to dampen cutaneous immune sensitization.

The mucosa-associated lymphoid tissue of the respiratory and enteric systems is in common in the tonsils and pharyngeal areas, which in humans, are collectively known as Waldeyer's ring. In the respiratory system, the epithelium of the nasal mucosa as well as the larynx, trachea, and bronchi are infiltrated to a variable extent with lymphocytes and to a lesser extent with neutrophils and macrophages. Mucosal infiltration is also more prominent above areas of submucosal lymphoid proliferation such as occurs in the tonsil and in bronchiolar-associated lymphoid tissues.² In general, the level of reactivity in these areas is related to the level of antigenic stimulation. Lymphoid proliferation in the tonsil is usually characterized by prominent germinal center formation, while in the lower lung, in conditions of health, the lymphoid tissue may be minimal and restricted to diffuse accumulations of cells between the bronchial epithelium and the supporting smooth muscle and cartilaginous plates (Fig. 52.14).²⁰ The impact of ambient air quality in terms of freedom from dust particles and airborne infectious agents is immediately apparent in the histology of laboratory animals. Animals maintained in facilities where the air is well filtered have minimal bronchiolar-associated lymph node tissue and few intra-epithelial lymphocytes. In contrast, animals maintained in less pristine circumstances char-

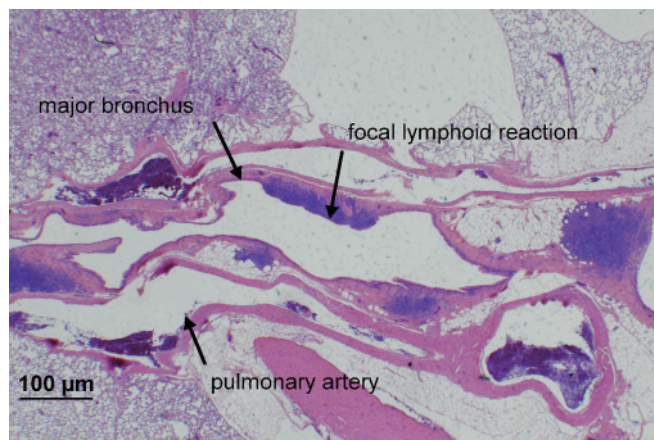


FIGURE 52.14 Lung from a normal rat with longitudinal section of a major bronchus. Note that the focal areas of lymphoid proliferation are both peribronchiolar and subepithelial. Hematoxylin & eosin stain; bar = 100 μ m.

acteristically have diffuse lymphoid proliferation in the proximal trachea and laryngeal areas with heavy and mixed cellular infiltration of the surface epithelium. In the lower lung, the lymphoid proliferation, which may contain germinal centers but is usually diffuse, may be of sufficient volume to partially occlude airways. As in other areas, the lymph nodes draining the lung at the base of the heart and in the caudal mediastinum bear the evidence of past environmental experiences including contact with smoke and other inhaled particulate matter.

The gut-associated lymphoid tissue forms a relatively continuous chain from the oral cavity to the anus in characteristic areas of proliferation. There are generally few lymphocytes in the epithelium and mucosa of the esophagus. Lymphoid proliferation with germinal center formation is not normal but relatively common beneath the gastric epithelium where lymphomas are relatively frequent in both humans and animals. In recent years, with discovery of the association between *Helicobacter* and human gastric ulceration, it has become apparent that most of the lymphoid tissue present in these cases is antigen dependent and rapidly recedes with long-term antibiotic therapy. Even more remarkably, cases of human gastric lymphoma in at least early stages of development appear to be antigen-dependent and may regress with antibiotic treatment alone. The organized areas of lymphoid tissue in the lower small intestine including the Peyer's patches are estimated to equal the thymus gland in young animals (Fig. 52.15). The epithelium overlying the intestinal germinal centers is generally devoid of goblet cells and villi, and glandular crypts are absent (Fig. 52.16). In these areas, the epithelium contains specialized cells with complex folding of the surface epithelium known as M cells.²³ M cells enfold lymphocytes within their membranes and function as antigen trephocytic cells able to metabolize particulate antigen to a level of digestion or nucleotide message that instructs the associated lymphocytes to specific immune reaction. Much of the immunoglobulin

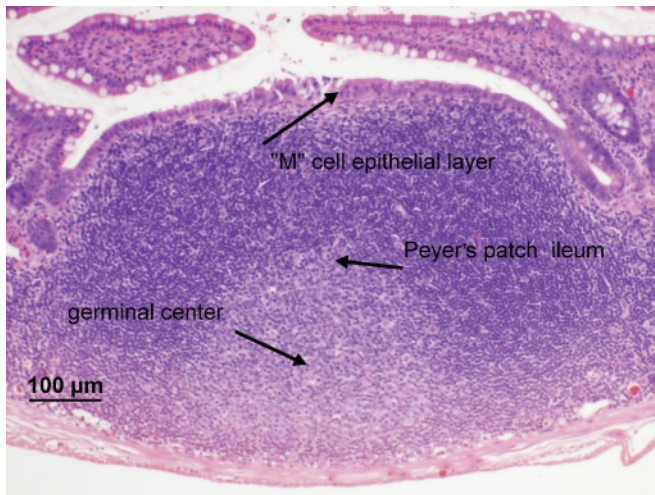


FIGURE 52.15 Lower small intestine of normal rat. A focal submucosal proliferation of lymphocytes in a dome area characterized by absent villi and modified epithelium to assist antigen recognition and sorting. Hematoxylin & eosin stain; bar = 100 μm .

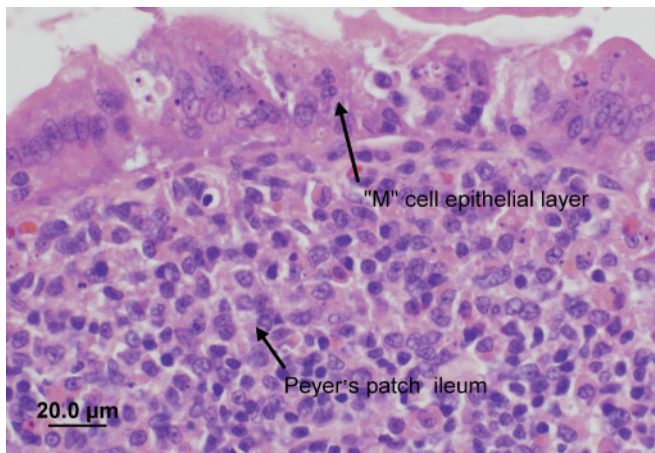


FIGURE 52.16 Details of Figure 52.15. The epithelium above the lymphoid follicle is modified to contain M cells that have deeply folded cellular membranes that enclose intraepithelial lymphocytes. The M cells are capable of uptake and of processing antigen from the intestinal lumen and providing immune instruction to maturing lymphocytes. In this area there are lymphocytes in the epithelium undergoing apoptosis presumably similar to the selection process in node germinal centers. Hematoxylin & eosin stain; bar = 20 μm .

produced in these areas is IgA-type with luminal and mucosal concentrations higher than that in blood.

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Disorders of the Spleen

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Spectrum of Spontaneous Splenic Disease in Animals
 Diagnostics
 Disorders of the Spleen
 Developmental Defects
 Circulatory Disturbances – Infarction
 Traumatic Lesions
 Infectious, Inflammatory, and Immune Disorders
 Infectious agents that affect the spleen
 Erythrotrophic pathogens
 Lymphotrophic pathogens
 Other pathogens affecting the spleen

Inflammatory and immune disorders affecting the spleen
 Toxic Injury
 Degenerative Conditions
 Splenomegaly and Hypersplenism
 Splenic histiocytosis
 Fibrohistiocytic nodules
 Splenic neoplasms
 Asplenia and Hyposplenism
 Summary

Acronyms and Abbreviations

BSE, bovine spongiform encephalopathy; BVD-MD, bovine viral diarrhea-mucosal disease; GDV, gastric dilation and volvulus; IMHA, immune-mediated hemolytic anemia; RBC, red blood cell.

The spleen serves many important roles in mammals. These include the production of cells in fetal and adult life, phagocytosis and opsonization of particulate materials and pathogens, filtering of blood, storage and release of blood cells to meet physiologic demands, participation in the recycling and metabolism of iron, and immune reactivity. The spleen is critically important in defense against a variety of pathogens. A detailed review of anatomy and physiology of the spleen is found in Chapter 52.

SPECTRUM OF SPONTANEOUS SPLENIC DISEASE IN ANIMALS

There is a large amount of published information on disorders of the spleen in humans, but considerably less for spontaneous diseases of the spleen in domesticated animals. Good data on the prevalence of disease is available for dogs and cats. In two papers, Spangler and Culbertson^{53,54} surveyed neoplastic and non-neoplastic splenic lesions in dogs and cats. Table 53.1 describes major neoplastic and non-neoplastic lesions in samples of dog spleens from three different sources. These data show that non-neoplastic disease accounts for approxi-

mately 60–80% of splenic lesions in dogs, depending on the source of the sample. A number of lesions of the canine spleen can be considered uncommon, occurring in 3% or less of samples submitted for diagnostic evaluation (Table 53.2).

Spangler and Culbertson also published data on the incidence of neoplastic and non-neoplastic lesions in the spleen of cats. Their data were formulated from evaluation of 455 specimens sent to the diagnostic laboratory of a teaching hospital (Table 53.3).⁵⁴ It is clear that in cats, neoplastic disease of the spleen is significant and that hyperplasia of various elements is noteworthy. The relative importance of a diagnosis of splenic congestion is problematic, given that many specimens collected from either species may have been done with benefit of anesthetic agents or from animals euthanized with barbiturates.

We have conducted a comprehensive review of the literature on splenic and hemolymphatic disease in horses.⁴⁴ Single and small cohort case reports on splenic rupture, abscessation, and infarction have appeared over the past 100 years, but given the paucity of this information, it appears that splenic disease is rarely a problem in horses. Horses, as dogs, show splenic enlargement when euthanized with barbiturate overdose, although to a lesser degree.

TABLE 53.1 Prevalence of Major Lesions Seen in the Splens of Dogs Submitted for Evaluation

Diagnosis	Group 1 ^a (n = 1,372)	Group 2 ^b (n = 92)	Group 3 ^c (n = 105)
Splenic hematoma	10%	19%	
Hyperplastic nodules	23%	6%	59%
Hematoma/nodules	10%	19%	
Hemangiosarcoma	10%	24%	9%
Neoplasm, not hemangiosarcoma	11%	21%	22%
Extramedullary hematopoiesis	6%		
Congestion	7%	2%	
Lymphoid hypoplasia	4%		

^aGroup 1. Specimens submitted to a diagnostic laboratory for evaluation by referring veterinary practitioners.

^bGroup 2. Specimens obtained by splenectomy at a teaching hospital.

^cGroup 3. Specimens obtained from a colony of Beagle dogs studied throughout their lifetime.⁵³

TABLE 53.2 Uncommon (3% or less of diagnoses) Non-Neoplastic Lesions in the Canine Spleen from 1,372 Submissions to a Diagnostic Laboratory of a Teaching Hospital⁵³

Diagnosis	% of Total Submissions
Accessory spleen	3%
Siderotic plaques	3%
Infarcts	1%
Arterial thrombosis	1%
Torsion	<1%
Splenitis	<1%
Abscess	<1%
Amyloidosis	<1%
Sinusoidal telangiectasis	<1%
Necrosis/hemorrhage	<1%
Fibrosis	<1%
Capsulitis/peritonitis	<1%
Capsular mineralization	<1%
Traumatic rupture	<1%
Necrosis	<1%

Cattle and other ruminants have splenic involvement with a variety of infectious, inflammatory, and immune-mediated diseases (discussed more fully below). Splenic changes frequently accompany systemic granulomatous disease and parasitic diseases, these changes being reflective of a splenic response to pathogens. Several cases of splenic disease in cattle due to exposure to plant or environmental toxins are reviewed. Within the past 10 years, the importance of the spleen as a potential reservoir for prions has been studied both for the scrapie agent in sheep and for determining the role of the spleen in the pathogenesis of bovine spongiform encephalopathy.

Rodents, dogs, and non-human primates are sometimes used as translational models for human diseases.^{17,18,32,45} Dogs, especially, are widely used as models for traumatic injury to the spleen, although the validity of the dog as a model might be questioned, given the

TABLE 53.3 Prevalence of Major Splenic Diseases in Cats obtained as Diagnoses from 455 Specimens Submitted to a Teaching Hospital Diagnostic Laboratory⁵⁴

Diagnosis	% of Total Submissions
Neoplastic disease	54%
Congestion	9%
Lymphoid hyperplasia	7%
Lymphoid hypoplasia/atrophy	7%
Capsulitis/peritonitis	5%
Extramedullary hematopoiesis	4%
Accessory spleen	4%
Hyperplastic nodule	3%
Splenitis	2%
Infarct	1%
Necrosis	1%
Lymphoid necrosis	1%
Reticuloendothelial hyperplasia	1%
Fibrosis	<1%
Amyloidosis	<1%

low incidence of traumatic splenic rupture in dogs. Likewise, rodents and dogs have been used to study the effects of splenectomy; however, dogs appear to be rather resistant to post-splenectomy sepsis.¹⁵ Unfortunately, there have been few studies of the pathobiology of this apparent resistance.⁶² Non-human primates are primarily used in laboratory settings and there is a paucity of literature on spontaneous splenic lesions of wild non-human primates.

DIAGNOSTICS

Studies over the past 10 years have resulted in several useful techniques for evaluation of normal and abnormal spleens. Several improvements have been made to standard and contrast enhanced splenography, ultrasonography, and to histopathologic methods of evaluation. Szatmari and co-workers⁵⁸ and Ohlerth and co-workers³⁷ described an ultrasonographic method, contrast harmonic imaging, to study the anatomy and kinetics of movement of a microbubble contrast agent in the spleen of normal dogs. The patterns of distribution and elimination of the agent were not adversely affected by age, gender, blood pressure, or blood composition variables. Rossi and co-workers⁴⁷ studied focal benign and malignant lesions, sonographically, in 26 dogs and 2 cats and correlated ultrasonographic interpretations with histopathology and cytology. They were able to distinguish disease-related ultrasonographic appearance for several types of malignancies. This confirmed earlier studies conducted with similar methods in 29 dogs and 3 cats.⁴

Cytologic interpretation of the spleen is becoming more commonplace. Christopher¹³ presented a comprehensive overview of the use and interpretation of fine-needle aspirates in the diagnosis of splenic disorders. However, a study of ultrasound-guided splenic

biopsies by Ballegeer and co-workers⁴ showed a relatively low rate (61%) of correlation between fine-needle aspiration biopsy diagnosis and histopathologic diagnosis. For non-neoplastic conditions, cytologic and histopathologic diagnosis agreed 11 out of 14 times. For neoplastic lesions, the cytologic diagnosis agreed with the histopathologic diagnosis in 8 out of 17 times.

DISORDERS OF THE SPLEEN

Developmental Defects

A rare congenital form of asplenia (familial isolated congenital asplenia) has been described in humans associated with mutations in connexin 43 and ZIC3 genes.^{8,20} A mutation in the Hox11 gene produces isolated congenital asplenia in mice.⁴⁰ Common clinical problems seen in humans with asplenia or hyposplenism include a wide variety of vascular, autoimmune, and thrombotic diseases.^{21,64} There have been few published reports of asplenia in domesticated animals.

Circulatory Disturbances – Infarction

In all mammals, the spleen is abundantly vascularized and has an anatomically unique and redundant circulation (see Chapter 52).² It is relatively resistant to circulatory compromise. Infarction of the spleen, from partial or complete occlusion of the splenic blood supply, can result in loss of splenic tissue and function. There are many causes. The presence of any occlusive bodies (e.g. thromboemboli, bacterial plaques, fat, air) that gain access to the circulation can cause splenic infarction. In ruminants, abscesses of the liver and mammary gland may erode the walls of major vessels in these organs, leading to septic embolic showers that infarct many organs, including the spleen. The enlargement of lesions in the mesentery, omentum, or in tissues and organs within the peritoneum (neoplasms and abscesses) are frequent causes of compression of the blood supply to the spleen, resulting in infarction. Partial blood flow and collateral circulation are important determinants in survival of splenic tissue after circulatory compromise. Wright and co-workers⁶⁵ introduced 20–500 μm microspheres into the circulation of dogs to investigate non-surgical methods for treatment of splenic masses. Dogs suffered few acute effects and within 6 weeks marked splenic fibrosis was noted.

Monsivais and co-workers³⁴ experimentally “infarcted” the spleens of 20 cats using total splenectomy. They then crushed and debrided a portion of the spleen, and reimplanted them with microvascular anastomosis after periods of one to several hours. Portions of the spleen that had adequate restoration of circulation returned to normal morphology and function, while those with residual circulatory deficits underwent atrophy by 8 weeks. This work demonstrated that restoration of circulation was requisite for function and this has direct implications in splenic infarction management in domesticated animals.

Gastric dilation and volvulus (GDV) in dogs frequently results in compromise of the blood supply to the spleen and can be a major cause of splenic infarction.^{9,25} In a study of 295 cases of GDV, splenic torsion occurred at a rate of 20%.⁹ Large breed and deep-chested phenotype are predisposing factors for torsion.³⁶ Splenectomy is recommended rather than de-torsion.⁶³ Release of myocardial depressant ions (potassium), microemboli, and bacterial toxins are all undesirable consequences of de-torsion. Complications associated with primary or secondary splenic torsion associated with GDV include cardiovascular compromise, hemorrhage, and coagulation disorders.⁵

Traumatic Lesions

Abdominal trauma can damage the spleen, leading to hemorrhage, hemoabdomen, and death. Common causes of trauma, including falling, impact with blunt objects (automobiles), and penetrating wounds (projectiles) all have been associated with splenic fracture or rupture. The spleen is generally protected from the direct effects of blunt trauma by intervening structures in the body wall, the cushioning effect of intraperitoneal fat, and the omentum. A complex but efficient system of ligaments and vessels hold its position.⁶ The fibroelastic capsule that invests the spleen also protects it from trauma. As reported by Spangler and Culbertson,^{53,54} rupture of the spleen occurs infrequently in dogs and cats. Splenic rupture in horses following episodes of falling, impalement, and dynamic force has been reported.⁴⁴

While the normal spleen is highly resistant to rupture, the diseased spleen is not. Splenic hemangiosarcoma may breach or compromise the splenic capsule. Uncontrolled bleeding from ruptured neoplastic tumors frequently leads to hemoabdomen and collapse of affected animals. Likewise, non-neoplastic tumors may compromise the integrity of the spleen and also lead to rupture and bleeding. The friability of the spleen that accompanies viral, protozoal, and bacterial infections can lead to splenic rupture.⁴⁹ Mice infected with *Plasmodium berghei* show splenic enlargement that can predispose to splenic degeneration and rupture.⁴¹

Accessory spleens and small splenic remnants following omental implantation are routinely recognized as incidental findings at post-mortem examination of animals. There are several theories concerning their origin. Some accessory spleens and remnants are thought to represent ectopic development of tissue during embryogenesis. Implantation of small pieces of spleen in the omentum, following splenic trauma, have been recognized frequently during necropsy, and it is theorized that fragments dislodged during the traumatic event are encapsulated and vascularized by omental tissue shortly after injury. Experimental studies in dogs showed that small splenic implants readily vascularize in the omentum and sustain some functions of normal spleens.^{50,61}

The differentiation of accessory splenic nodules and splenic remnants is important diagnostically when

encountering dogs with splenic hemangiosarcoma. Histological benign accessory spleens and remnants may be confused with implantation metastases. In these cases, samples of suspect “metastases” should be harvested and evaluated histopathologically.

Infectious, Inflammatory, and Immune Disorders

Infectious Agents That Affect the Spleen

Erythrotoxic Pathogens Pathogens that target red blood cells (RBCs) including *Mycoplasma* sp. (previously termed *Haemobartonella*), *Babesia* sp., *Plasmodium* sp., *Anaplasma* sp., *Theileria* sp., and *Eperythrozoon* sp. all produce splenic lesions consistent with the spleen’s role in the management of degenerate RBCs.^{14,29,44,57} All are important pathogens of domestic animals or humans, causing significant economic loss, morbidity, and mortality. Many are transmitted through intermediate vectors. A few merit further discussion.

Mycoplasma felis is an important acute and chronic disease in cats (see Chapter 31).⁴⁴ The spleen plays the major role in extravascular destruction of infected RBCs. During the course of disease, splenomegaly is a reflection of increased demand for splenic function. Various studies have considered the importance of the spleen in either participating in the pathogenesis of clinical signs or, conversely, in maintaining resistance to infection in cats. Although it was believed that most splenectomized cats would develop hemobartonellosis,¹⁸ this has not been demonstrated in experimental studies.

Bartonella infection in cats is considered to be one of a number of related emerging zoonotic diseases.⁷ At least five separate *Bartonella* species infect cats as intracellular parasites in RBCs and vascular endothelium, and separate species of the genus are widely distributed in other mammals. In the spleen, liver, and lymph nodes of infected cats, this pathogen can incite granulomatous inflammation. Subclinical disease has been reported and while not frequent, thrombocytopenia, neutropenia, and lymphocytosis have been detected in some infected cats. Definitive diagnosis of infection can be made with molecular probes (polymerase chain reaction). Other causes of splenic granulomatous disease in cats, including blastomycosis,¹⁰ must be considered when evaluating cats with splenic nodules.

Lymphotropic Pathogens Some pathogens exert their systemic effects by colonizing lymphoid tissues, including splenic white pulp. In these tissues, the response to infection can vary from tissue destruction to passive proliferation of the infectious agent. Numerous studies have documented the importance of silent infection and carrier states in the propagation of some infections. In the past decade, several lymphotropic pathogens that affect splenic morphology and function have been more studied. Recent experimental studies of *Theileria parva*, the causative agent of East Coast fever of cattle in Africa, show that the tropism of the parasite for lymphoid tissue results in massive destruction of lymphocytes

and splenic germinal centers.³¹ Spleen collected from calves showed enlargement, caseous necrosis, and degeneration of both white and red pulp. Bovine viral diarrhea-mucosal disease (BVD-MD) appears to cause lympholysis in the spleen, lymph nodes, Peyer’s patches and thymus. These changes were most prominent in younger animals and in those with more prominent clinical signs.³⁰ Experimental bovine herpesvirus-1 infection was associated with lympholysis in spleen and other lymphoid tissues and vascular endothelial degeneration and necrosis of tissues in aborted fetuses from heifers.⁴²

Other Pathogens Affecting the Spleen Public attention has been focused on the transmission of prions in scrapie and bovine spongiform encephalopathy (BSE). Sheep inoculated experimentally with brain homogenate from BSE-positive cattle had accumulation and expression of scrapie-associated prion protein in spleen, gut-associated lymphoid tissues, and lymph nodes 6–9 months after exposure.⁶⁰ Subsequent to localization and expression of prion protein in lymphoid tissues, the spread of protein to neural tissue via gut-associated parasympathetic and sympathetic nerve roots was documented. This work underscores the complexity of infection and transmission of prions in disease, and a potentially facilitating role of the spleen in the pathogenesis of infection.

Infection of dogs with *Leishmania infantum* is widespread, especially in warmer climates, where vectors for the disease are commonplace (see Chapter 19). Various stages of the parasite are present in tissue including the spleen, liver, bone marrow, and skin.⁴⁸ The amount of parasitic DNA was consistently higher in the spleen than bone marrow, indicating a high level of tissue tropism in this infection.⁴³

Inflammatory and Immune Disorders Affecting the Spleen

Amyloidosis is a relatively uncommon lesion of the spleen of dogs and cats (see Tables 53.2 and 53.3).^{53,54} Amyloid deposition around arterioles is the most frequent presentation and such deposits, like amyloid in any tissue, can lead to progressive tissue hypoxia and dysfunction. Yamada and co-workers⁶⁶ studied the occurrence and distribution of AA amyloid in tissues of 25 cows diagnosed with systemic AA amyloidosis. Fibrils leading to tissue dysfunction were seen in the spleen as well as many other tissues. Ten cows had evidence of chronic inflammatory diseases including mastitis ($n = 6$) and pneumonia ($n = 4$), while a focus of inflammation could not be conclusively identified in other cattle.

In a recent study of the relationship of leukocytosis and tissue pathology in immune-mediated hemolytic anemia (IMHA) of dogs, ischemic necrosis of the spleen, liver, kidney, heart, and lungs were seen in varying degrees in 34 dogs and was attributed to thromboembolism and anemic hypoxia.³³ The presence and

severity of lesions in tissues was correlated to the severity of antemortem leukocytosis.

A single case report of eosinophilic syndrome involving the spleen of a dog has been reported.³ The affected dog showed a mature, infiltrative eosinophilia of the spleen, musculature, and lymph nodes, as well as heart and lungs. A leukemoid eosinophilic response was present in the blood. The cause of this disease was not determined.

Toxic Injury

The spleen is not an organ that is typically the site of direct toxic injury, with the exception of lympholysis associated with exposure to corticosteroids, non-steroidal anti-inflammatory agents, and cancer chemotherapies. However, there have been recent reports of splenic lesions associated with exposure to citrus pulp, hairy vetch, and lead.

Iizuki and co-workers²⁶ performed detailed studies of seven cows that presented with urticaria, loss of lactation, and death after ingestion of a diet rich in citrus pulp. Severe granulomatous disease, with a lymphoplasmacytic and giant cell infiltrate, was seen in kidneys and spleen. Evaluation with immunohistochemical markers showed infiltrations with CD3+ T cells associated with a systemic hypersensitivity reaction to components of the diet.

Hairy vetch poisoning was associated with splenic lesions in eight herds in Brazil.¹⁶ Granulomas, presumably associated with organ dysfunction, were observed in spleen and many other tissues. Granulomatous inflammation of the myocardium was considered the most significant clinical lesion.

Splenic hemosiderosis, as a consequence of exposure to high levels of environmental lead, was seen in a herd of Nelore cows in Brazil.²⁸ Thirty-five died within 2–7 days of exposure to fumes and forage contaminated with lead from a car battery recycling facility. Affected cattle developed secondary hemosiderosis of the spleen, liver, and kidney associated with lysis of RBCs.

Degenerative Conditions

Splenic atrophy has been reported in many aged horses.⁴⁴ Horses were euthanized for health problems ranging from chronic lameness and colic to failure to thrive. Age-associated thickening of the splenic capsule, focal capsular fibrosis, hypoplasia of red and white pulp, and parenchymal fibrosis were the most frequent histopathologic findings. Similar age-associated changes have been noted in the spleens of dogs, cats, and several species of ruminants.

Splenomegaly and Hypersplenism

There are many causes of splenomegaly. Pathologists most frequently encounter splenomegaly as a consequence of euthanasia in which barbiturate overdose is used. Another frequent cause is nodular proliferation. Many of these nodules are benign, while some are asso-

ciated with tumors of the spleen, including hemangioma, hemangiosarcoma, and malignant lymphoma. The spleen is a frequent site for formation of granulomas associated with infectious organisms, such as *Mycobacterium* sp., fungi, and yeasts.

Hypersplenism is defined in human medicine by four criteria including peripheral cytopenia, improvement of cytopenia following splenectomy, reactive hyperplasia of the bone marrow, and splenomegaly.⁵⁶ Hypersplenism is not well defined as a disorder in veterinary medicine.

Splenic Histiocytosis

In two papers, Spangler and Kass^{55,56} described several different types of lesions leading to splenomegaly in dogs. A distinctive form of splenic histiocytosis, associated with cytopenia, vomiting, depression, and fever was recognized by the analysis of specimens from 65 dogs.⁵⁶ The disease was most frequent in Golden Retriever, Labrador Retriever, and Shepherd-type dogs; a mean age of 8 years and a range of 2–15 years was reported. These breeds of dog also are some of the breeds at highest risk for the development of canine malignant lymphoma and hemangiosarcoma. Histologically, the spleens showed varying degrees of diffuse extramedullary hematopoiesis (termed myeloid metaplasia), reactive/proliferative histiocytosis, erythrophagocytosis, and vascular thrombosis leading to splenic infarction. In some cases, distinctive giant cells of histiocytic origin were observed. No consistent alterations in hematology or serum chemistry profiles were predictive of the course of disease in these patients. These authors hypothesized that these lesions may represent parts of a continuum of splenic histiocytosis that progresses to histiocytic neoplasia.

Fibrohistiocytic Nodules

In a separate paper Spangler and Kass⁵⁵ propose that fibrohistiocytic nodules form a continuum between splenic nodular lymphoid hyperplasia and malignant fibrous histiocytoma. Specimens of splenic lesions from 98 dogs were submitted for evaluation by a diagnostic pathology service. Microscopic evaluation of specimens had a high prevalence of fibrohistiocytic cells, demonstrated by immunohistochemical staining for vimentin and desmin. Variable numbers of lymphocytes, plasma cells, and hematopoietic cells formed the remainder of the nodules. About half of dogs with fibrohistiocytic nodules died within 12 months of the time of diagnosis; about half of the cases were felt to die as a direct result of the splenic lesions. No consistent alterations in hematology or serum chemistry profiles were predictive of the course of disease in these patients.

Splenic Neoplasms

In the dog, splenic neoplasms are considered to be a major cause of splenomegaly, although splenic hematoma and reactive hyperplasia are the most

frequent causes of splenomegaly.⁵³ Hemangiosarcoma is the most frequent tumor of the spleen, its frequency being as high as all other splenic tumors combined. Haematopoietic tumors (e.g. malignant lymphoma and malignant histiocytosis, hemangiomas) are other important tumors of the spleen of dogs. In the cat, primary and metastatic neoplasia account for one-third of all splenic lesions. In this species, however, the frequency of occurrence differs from the dog, with mast cell tumors being the most frequent and hemangiosarcomas a less frequent tumor type.⁵⁴

Histiocytic sarcoma, formerly called malignant histiocytosis, is a tumour originating from myeloid dendritic antigen-presenting cells (see Chapter 73).^{1,46,51} A few cases of malignant histiocytosis in the cat have been reported.¹⁹ Discernible splenomegaly is seen in approximately 50% of dogs. A variant of the histiocytic sarcoma is the hemophagocytic histiocytic sarcoma; this was described in several dogs (see Chapter 73).³⁵ Diffuse splenomegaly was the main hallmark.

In both the alimentary and the multicentric form of malignant lymphoma in dogs, cats and cattle, the spleen can become involved in the disease process and become enlarged due to infiltration with neoplastic lymphoblasts and lymphocytes. Single involvement of the spleen in malignant lymphoma is uncommon.⁵⁹ The splenic form of lymphosarcoma in horses can produce dramatic splenomegaly and leukemia and is considered rare.⁴⁴

Splenic hemangiomas and hemangiosarcomas occur more frequently in dogs (prevalence 0.3–2%^{38,39}) than in any other species. Especially prone to develop these tumors are middle-aged and older large breed dogs, with German Shepherd dogs and Golden Retrievers having an unusually high incidence.²⁶ These tumors typically appear as large, solitary friable nodules that penetrate the capsule and easily bleed uncontrollably. Multiple nodules also may be seen. In dogs with hemangiosarcoma, regenerative anemia and thrombocytopenia are frequently observed, due to bleeding within and from the tumor. Some dogs die from hemorrhage after rupture of splenic hemangiosarcomas. Many tumors have metastasized to liver and lung by the time they are diagnosed. Although surgical resection of hemangiosarcoma has been the mainstay of treatment, survival times have been short. Median survival time after surgery alone is between 19 and 65 days.^{11,24,26,39} Clinical staging may have some prognostic value. In one study, dogs with splenic hemangiosarcoma and hemoperitoneum had a median survival of 17 days. In contrast, the median survival of dogs with hemangiosarcoma, splenectomized before developing hemoperitoneum, was 121 days.³⁹ However, two other studies could not confirm this observation.^{11,26} Chemotherapy with doxorubicin (30 mg/m² every other 3 weeks) and cyclophosphamide (50–75 mg/m² during three days, every other 3 weeks) as an adjuvant therapy to surgery in dogs with splenic hemangiosarcoma has been reported to prolong median survival to 180 days.^{22,52} Preliminary results with continuous orally administered low-dose chemotherapy using a combination of cyclophosphamide, etoposide, and piroxicam as adjuvant therapy for dogs

may be an effective alternative to conventional high-dose chemotherapy for treatment of dogs with hemangiosarcoma.²⁷

Metastatic disease can also occur in the spleen, but is not common. In dogs and horses with myelogenous leukemia, the spleen can be markedly enlarged, due to filling of splenic sinusoids with neoplastic cells.¹²

Asplenia and Hyposplenism

Hyposplenism and asplenia are broad terms used to describe decreased or absent splenic function. Hyposplenism can be associated with decreased (or absent) splenic size (described above), but can also occur in the face of splenomegaly. These conditions have many causes. It is well recognized that asplenic human patients (after splenectomy) and those with hypofunctional spleens are highly susceptible to infection.^{23,64} In dogs and cats, loss of splenic function can accompany therapeutic splenectomy (surgical intervention in trauma or neoplastic disease), suppression with drugs (e.g. corticosteroids, cancer chemotherapy), exposure to toxic chemicals, radiation, and with interruption of the splenic blood supply (primary infarction or secondary to torsion). In general, splenectomized dogs and cats appear to have a lower incidence of sepsis than do humans.

SUMMARY

Considerable study of splenic disorders has occurred in the past decade. Although this has improved our knowledge of many disease conditions, the arbitrary line dividing non-neoplastic and neoplastic diseases has become blurred, especially for histiocytic proliferative conditions. We expect continued enthusiasm for study of the role of the spleen in health and disease. Finally, veterinarians, when recognizing splenic disease, frequently remove the spleen. While this intervention is certainly indicated with localized neoplastic disease, it may warrant rethinking when dealing with trauma. In essence, the spleen is there for a reason and we should try to preserve it.⁶²

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Systemic Lupus Erythematosus

LUC S. CHABANNE

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Genetic Factors	Feline SLE
Environmental Factors and Infectious Agents	Equine SLE
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Acronyms and Abbreviations

ANA, antinuclear antibody; ACR, American College of Rheumatology; APC, antigen-presenting cell; CBC, complete blood count; CD, cluster of differentiation; CIC, circulating immune complex; CTLA-4, cytotoxic T lymphocyte associated protein 4; DLA, dog leukocyte antigen; dsDNA, double-stranded DNA; ELISA, enzyme-linked immunosorbent assay; ENA, extractable nuclear antigen; FeLV, feline leukemia virus; FIV, feline immunodeficiency virus; H, histone; Ig, immunoglobulin; IMHA, immune-mediated hemolytic anemia; LE, lupus erythematosus; MHC, major histocompatibility complex; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; RBC, red blood cell; RNP, ribonucleoprotein; SLE, systemic lupus erythematosus; SS-A, Sjögren syndrome antigen A; TCR, T cell receptor.

Systemic lupus erythematosus (SLE) is a chronic, recurrent inflammatory disorder characterized by autoantibodies directed against self-antigens, immune complex formation, and immune dysregulation. SLE causes damage of multiple organs and leads to a wide range of clinical manifestations. Previously believed to be a rare disease, there has been an increase in awareness and education about SLE since the 1960s. In the United States and Northern Europe, the prevalence in humans is approximately 1 case per 2000 people, but varies according to location and ethnicity.

In animals, a number of inbred strains of mice (e.g. the New Zealand black/white strain) spontaneously develop lupus-like syndromes, and this has allowed intensive study of disease mechanisms. Systemic lupus erythematosus is recognized in monkeys,¹ dogs, cats, and horses. SLE-like disorders have also been reported in poikilothermic species, such as snakes and iguanas.¹³

This chapter will discuss SLE in dogs, cats and horses. Considered as an infrequent disease in the dog, many studies have been published on canine SLE since its initial description in 1965,²⁰ while feline and equine SLE

are rare. Thus, most information presented in this chapter derives from canine studies. Recently, an experimental canine model of SLE has been developed, and could be interesting for a better understanding of this complex disease and evaluation of new therapeutic approaches.⁹

RISK FACTORS

The cause of SLE remains unknown, although both genetic and environmental factors appear to play an important part in the induction and perpetuation of the disease.

Genetic Factors

Susceptibility loci with confirmed linkage to SLE have been identified in humans and mice. These loci contain genes encoding molecules involved in immune response and apoptosis. Most of these genes are associated with the major histocompatibility complex (MHC). The multitude of distinct genetic associations suggests a complex genetic predisposition for the disease, perhaps explaining the variable courses and organ system involvement.

The influence of genetic background in canine SLE is supported by studies of inbred lines and evidence of familial aggregation.^{17,21,25,35} Systemic lupus erythematosus is associated with the dog leukocyte antigen (DLA): the MHC class I DLA-A7 allele confers risk for disease expression, whereas dogs possessing the DLA-A1 and DLA-B5 (a MHC class II allele) are at decreased risk.³⁵ Dogs with a specific complement C4 allotype, a class III gene, may be predisposed to a lupus-like syndrome characterized by non-erosive polyarthritis, pyrexia, and antinuclear antibodies (ANAs).¹¹

Feline SLE may occur more frequently in purebred cats, particularly those with a Siamese or Persian background, which also suggests a genetic influence in this species.

Environmental Factors and Infectious Agents

Other factors capable of causing the disease include environmental factors and infectious agents. A higher risk of developing ANA or SLE in pet dogs owned by human patients with SLE has been occasionally reported and supports the idea that a common environmental factor or zoonotic agent may have a role in causing SLE.^{8,19}

Light

Ultraviolet radiation is the most obvious environmental factor linked to SLE. Sunlight is known to be capable of triggering, or aggravating, SLE in humans, dogs, and cats. A photosensitive rash is a criterion of the American College of Rheumatology (ACR) for the diagnosis of the disease.³⁴

Infectious Agents

While a microbial etiology for autoimmune diseases remains a popular theory, supportive evidence in SLE is limited. A role for viruses in the induction of canine SLE has been proposed. Antigens that cross-react with C-type retroviral particles have been identified on the cell membrane of blood lymphocytes from affected dogs, and cell-free extracts were able to transfer the serological abnormalities of SLE from affected to normal dogs.²⁹ However, these findings have not been widely corroborated and no direct isolation of virus from canine tissues has been described. In cats, the retroviruses feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV) can induce disease with similarities to SLE (e.g. immune-mediated hemolytic anemia, IMHA), and ANA develops in some cats infected with FeLV. However, most cases of feline SLE are seronegative for both viruses.²⁷

Geographic Influence

Canine SLE is rarely diagnosed in the United States, Northern Europe, and Australia, but seems more frequently recognized in Mediterranean countries. This may reflect an association with different factors including breed distribution, regional gene pools, sun exposure, and prevalence of infectious diseases (e.g. leishmaniasis).⁷

Drugs and Food

In humans, many drugs cause a variant of lupus called drug-induced lupus. The best known of these drugs are procainamide, hydralazine, and quinidine. Lupus-like syndromes have been induced by hydralazine in the dog,³ and propylthiouracil in the cat.² Methimazole has been associated with the development of ANA in cats, but clinical signs of SLE have not been reported.²⁸

Other possible factors implicated in humans include foods (celery, parsley) containing psoralens that could potentially increase photosensitivity, l-canavanine (found in alfalfa seeds, sprouts, and legumes) that is structurally related to hydralazine, hair dyes, exposure to vinyl chloride, and implantation of silicone polymers.

Sex Hormones

Since 90% of human patients with lupus are female, an important role for female hormones seems probable, but a protective role for male hormones or an effect of genes of the X chromosome is also possible. Such gender distribution is not clearly defined in the dog.

PATHOGENESIS

There is compelling evidence that the immune system plays a pathogenic role in SLE.³⁰ Systemic lupus erythematosus is the prototype multisystem autoimmune disease in which immune dysregulation leads to failure

of the mechanisms that normally prevent the development of self-response.

Induction of Autoimmunity

Breakdown in tolerance, either central or peripheral, is a fundamental process in autoimmune disease. Central tolerance involves bone marrow and thymic deletion of autoreactive lymphocytes (negative selection) and positive selection of T cells with low affinity for self-MHC. However, T and B cell clonal deletion is not absolute and self-reactive lymphocytes escape to the periphery where self-reactivity is regulated by other mechanisms (Fig. 54.1). Autoimmune disease occurs when these regulatory factors fail and allow activation of self-reactive T and B cells. Environmental stimulus (e.g. microbial infections or drugs) has the potential to initiate and perpetuate autoreactivity through cross reactivity and molecular mimicry, development of anti-idiotypic antibodies that cross-react with self antigens, development of altered self-antigens, the release of previously hidden antigens, and polyclonal stimulation of naturally-occurring autoantibody producing cells.

Autoantibodies

SLE patients develop a wide variety of autoantibodies (Table 54.1).^{5,24,26,32} Increased production of autoanti-

TABLE 54.1 Autoantibodies in Canine SLE^a

Autoantibody	Percent Positive
Antinuclear (indirect IF techniques)	95–100
Anti-dsDNA	<3
Anti-ENA	
Total	40
Sm	16
RNP	8
hnRNP G	20
Canine type 2	9
SS-A/Ro	4
Anti-histones	
Total	65
H1	8
H2A	22
H2B	20
H3	54
H4	54
Anti-nucleosome	7
Anti-phospholipids	ND
Anti-erythrocyte antibodies (Coombs' test)	17
Anti-IgG antibodies (rheumatoid factors)	20
Circulating immune complexes	75
IgG deposits on cutaneous biopsies	75

^aENA, extractable nuclear antigens; H, histone; ND, not done; RNP, ribonucleoprotein; SS-A/Ro, Sjögren syndrome A antigen (or its equivalent Ro).

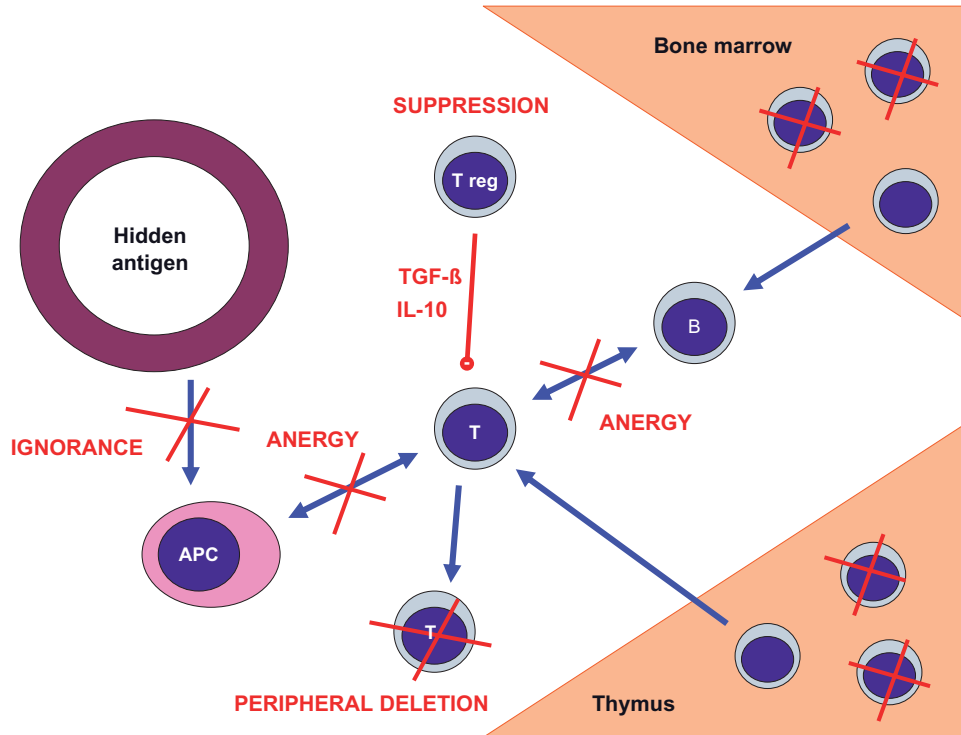


FIGURE 54.1 Potential mechanisms of immune tolerance: Autoreactive T and B cell clones that escape negative selection during maturation in the thymus or bone marrow, respectively, may be regulated in the periphery. Mechanisms involved to explain peripheral tolerance include: (1) ignorance of the antigen by the immune system (hidden antigen within cells or tissues and/or failure of antigen-presenting cells (APC) to present autoantigens to autoreactive T cells); (2) anergy (loss of co-stimulatory signals, down-regulation of receptors critical for T-cell activation); (3) peripheral deletion (apoptosis of autoreactive T cells due to recognition of autoantigen after release from the thymus); (4) suppression of autoreactive T and B cells by regulatory lymphoid populations (T reg, T-regulator cells).

bodies leads to immune complex formation and tissue damage from direct binding in tissues, immune complex deposition in tissues, or both.

Antinuclear Antibodies

Antibodies against the nuclear antigens are the most significant autoantibodies found in SLE. One proposed mechanism for development of ANAs involves a defect in apoptosis that causes increased cell death. During apoptosis, blebs of cellular material form on the surface of dying cells. Antigens that are normally buried within the cell are exposed on the surface of these blebs, and they may trigger an immune response (Fig. 54.2). These exposed antigens include nucleosomes (DNA and histone proteins), extractable nuclear antigens (ribonucleoproteins, RNP), and anionic phospholipids. The clearance of apoptotic debris is abnormal in patients with lupus. Normally, apoptotic cells are removed by phagocytes without causing inflammation. Macrophages from SLE patients, however, show defective phagocytosis of apoptotic cells, which as a result accumulate in tissues.

Unlike humans, dogs do not develop ANAs against native DNA (double-stranded DNA, dsDNA), and there is a restricted pattern of reactivity against the various individual histone antigens (H1, H2A, H3 and H4 polypeptides), while ANAs specific to nucleosome or histone-DNA complexes are rare.^{5,24,26} Canine ANAs also appear to have additional reactivities not found with human SLE sera (e.g. for the glycoprotein hnRNP

G).³¹ In contrast, the pattern of reactivity of other extractable nuclear antigens found in dogs is similar to that found in humans; for example, anti-Sm antibodies and anti-SS-A/Ro antibodies. Antibodies to the proliferating cell nuclear antigen (PCNA) have also been described in dogs.

ANAs mediate tissue inflammation and injury in SLE either by direct pathogenic effect (e.g. anti-SS-A/Ro activity against skin) or due to their association with antigens to form immune complexes either in situ or in circulation. Moreover, a wide range of abnormalities contributes to an impaired clearance of circulating immune complexes (CIC) in SLE (e.g. abnormal complement receptors, deficiency of complement components), and CIC are not effectively removed from the circulation. Thus, the net result is prolonged high levels of CIC, which become available for deposition in non-lymphoid sites such as kidneys, synovium, or skin. Inappropriate tissue deposition or formation in situ may result in complement activation, granulocyte chemotaxis, and release of mediators of inflammation leading to vascular injury (type III hypersensitivity) and tissue damage.

B Cell Response

Although ANAs are characteristics of SLE, many other autoantibodies are produced suggesting that affected patients have abnormalities in B cell function resulting in B cell hyperactivity.

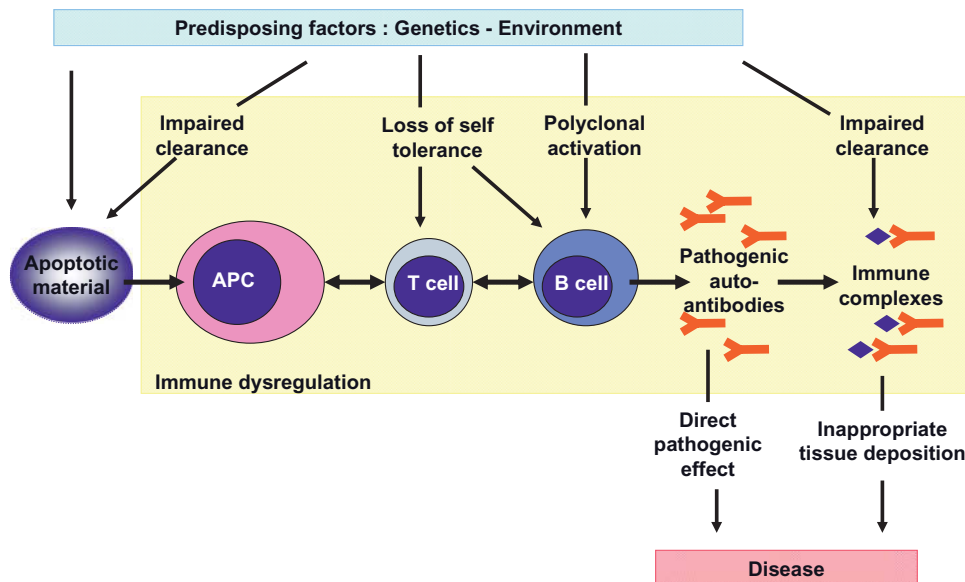


FIGURE 54.2 A simplified diagram of the immunologic events thought to occur in SLE leading to antinuclear antibody formation and immune complex deposition. Initiation of disease involves a combination of predisposing factors that give rise to accumulation of apoptotic debris and immune dysregulation. Antigens that are normally buried within the cells are exposed on the surface of the dying cell and trigger an immune response. Loss of tolerance toward self-antigens combined with polyclonal stimulation perpetuates autoantibody secretion. Autoantibodies may cause a direct pathogenic effect or lead to immune complex formation and tissue damage. Impaired clearance by the mononuclear phagocyte system promotes immune complex deposition. (Adapted from Rahman A, Isenberg DA. Systemic lupus erythematosus. *New Engl J Med* 2008;358:929–939, with permission.)

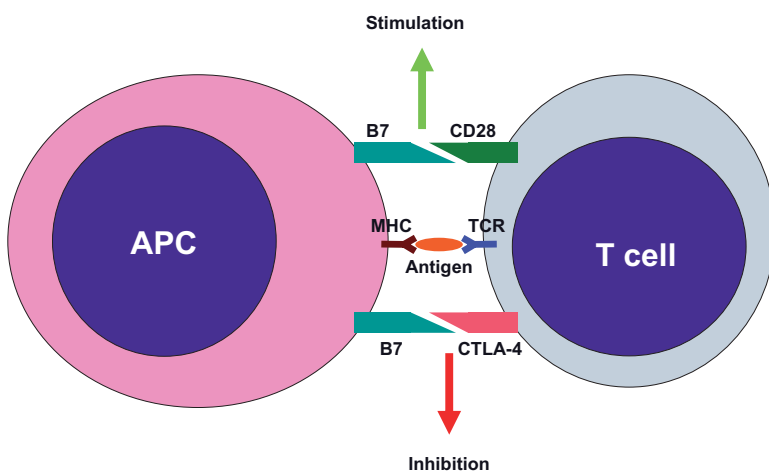
T Cell Defects

Abnormalities in T cell functions play a part in the induction and maintenance of B cell hyperactivity. T cell cytokines affect B cells by stimulating cell division, switching antibody production from IgM to IgG, and promoting a change in the molecular sequence of the secreted antibody so that it binds more strongly to the driving antigen. Thus, T cells help make possible the production of high-affinity IgG autoantibodies. Defective suppressor T cell function also can fail to regulate B cells.

Data from canine studies has shown immunologic alterations involving T cells and supports a defective suppressor T cell function: (1) a rapid decline in the level of a soluble thymic factor in canine SLE sera; (2) resistance to induced tolerance in some cases of canine SLE; (3) deficient concanavalin A-induced suppressor cell function; and (4) a decrease in the number of circulating CD8+ T cells as shown by studies on T-cell subsets.^{5,6} The extent of this last abnormality is directly related to the severity of the disease.

Canine Experimental Model

Recently, an experimental model of canine SLE has been developed.⁹ Systemic lupus erythematosus was induced in mongrel and mixed-breed dogs by immunization with heparan sulfate, the major glycosaminoglycan of the glomerular basement membrane. The dogs developed a lupus-like syndrome with proteinuria, skin lesions, and ANA. This model has been applied to gene therapy using a non-viral peptide vector encoding canine CTLA-4-Ig gene.¹⁰ Cytotoxic T cell-associated protein 4 (CTLA-4 or CD152) found on activated T cells is the ligand for CD80 (B7-1) and CD86 (B7-2) expressed on APC (Fig. 54.3). The CTLA-4-Ig fusion protein blocks the CD28-B7 interaction and thus directly targets the interaction between T cells and APC by inhibiting co-stimulation.



CLINICAL PRESENTATION

SLE is a chronic, multifaceted inflammatory disease that can affect every organ system of the body. SLE is protean in its manifestations and follows a relapsing and remitting course.

Hematological Disorders

A history of multiple cytopenias such as leukopenia, lymphopenia, anemia, or thrombocytopenia may suggest SLE, among other etiologies. Hemolytic anemia and thrombocytopenia were the main clinical signs together with nephritis cited in the initial description of canine SLE.²¹ In more recent canine studies, and in feline cases, hemolytic anemia is a moderately frequent finding as it is in human SLE, whereas thrombocytopenia is rare in dogs, cats, and humans. Coombs' test to denote antibodies on red blood cells (RBCs) and tests for anti-platelet antibody are used to demonstrate an immune-mediated mechanism (see Chapter 140). The Coombs' test is positive in 17% of canine SLE cases, even occasionally in the absence of clinical signs of hemolysis. A mild nonregenerative, Coombs' negative, anemia associated with chronic inflammation also can be present.

Leukopenia, due to lymphopenia, is not uncommon in SLE. During active disease, there is a marked lymphopenia, presumably secondary to the presence of anti-T cell antibodies. An analysis of canine lymphocyte subsets in the peripheral blood reveals a striking decrease in the CD8+ population, which is directly related to the severity of the disease. In contrast, although the percentage of CD4+ cells increases in SLE, the absolute number decreases. Consequently, the CD4:CD8 ratio may climb as high as 6 compared with a normal value of 2.3 in control dogs. Another important finding is the intense staining of T cells by activation markers.⁶

FIGURE 54.3 Interaction between a T cell and an antigen-presenting cell (APC). The APC binds antigen in a complex with a molecule from the MHC on its surface. This complex interacts with the T cell receptor (TCR). The effect on the T cells depends on the interaction between other molecules on the surfaces of the two cells. Two alternative interactions are shown: B7 with CD28, which is stimulatory, and B7 with cytotoxic T cell-associated protein 4 (CTLA-4), which is inhibitory. If the positive signal caused by the CD28-B7 interaction dominates, the T cell is activated, leading to cytokine release, B cell help, and inflammation. If the negative signal caused by the CTLA-4-B7 interaction dominates, activation is suppressed.

TABLE 54.2 Clinical Signs in Canine SLE

Signs	Percent positive
Fever	70–100
Non-erosive polyarthritis	80–90
Renal disorders	55–65
Muco-cutaneous lesions	45–60
Lymphadenopathy and/or splenomegaly	40–50
Hemolytic anemia	15
Thrombocytopenia	5–15
Leukopenia	15–20
Myositis	5–10
Pleuropericarditis	<10
Central nervous system disorders	<5
Polyneuritis	<2

Abnormalities in coagulation pathways can be identified in some patients, resulting in prolongation of the partial thromboplastin time, which fails to be corrected with the addition of normal plasma.^{22,33} These abnormalities are due to the presence of “lupus anticoagulant”, an antibody directed against phospholipids. In human patients, it defines the phospholipid antibody syndrome, which is associated with a thrombotic disorder.

Overview of the Disease

Canine SLE

Systemic lupus erythematosus is mainly recognized in middle-aged dogs. The clinical expression of canine SLE encompasses a wide range of clinical features (Table 54.2).^{5,12,15,32} Symptoms come and go unpredictably, and are observed successively rather than simultaneously as the disease evolves, except in advanced cases.

Constitutional manifestations such as loss of body mass, inappetence, fatigue and fever are frequent when the disease is active. Musculoskeletal symptoms are the most frequent primary signs recognized in the dog, usually manifesting as polyarthritis, whereas polymyositis is occasionally identified. Joints most frequently involved are the distal joints (e.g. carpi, tarsi, elbows, and stifles). The vertebral articular facets could also be affected and, later in the course of the disease, the temporo-mandibular joints. The arthritis is usually symmetrical, non-erosive, and non-deforming. The arthritis/arthralgia may be evanescent, and recurrent. Synovial fluid analysis shows an increased nucleated cell count and predominance of neutrophils. Renal involvement is frequent, but can be asymptomatic initially. Proteinuria, or microhematuria, is the earliest manifestation and may be the only presenting renal symptom. Immune-complex glomerulonephritis can range in severity from minor mesangial changes to diffuse proliferative lesions. Skin manifestations are highly variable and involve both cutaneous and mucous membranes. The lesions usually are characterized by photosensitization, with selective localization in areas exposed to sunlight (the

dorsal region of the anterior limbs, the face with lips, nose-pad, external ears in dogs with erect ears), and also in areas of thin skin poorly protected by the hair coat (axilla, inguinal region, ventral abdomen). Oral ulcers are infrequent but palatal ulcers are highly suggestive of SLE. Subacute cutaneous lupus erythematosus in humans defines a subgroup of SLE patients characterized by photosensitivity, non-scarring dermatitis, mild systemic features, and a low prevalence of nephritis. These patients are frequently ANA negative, but have antibodies to SS-A/Ro. Recent studies provide evidence supporting the hypothesis that vesicular cutaneous lupus erythematosus in the Rough Collie and Shetland dog is a homologue of subacute cutaneous lupus erythematosus.¹⁸ Lymphadenopathy or splenomegaly are common presenting signs (50%). In some cases, there is acute extensive facial or hemifacial edema. Cytological examination of enlarged lymph nodes shows plasma cell hyperplasia with an increase in small nucleolated cells or eosinophilic lymphadenitis. Cardiopulmonary involvement is not commonly documented in canine SLE. Features include serositis (pleuritis, pericarditis), and myocarditis.²³ Also neurological manifestations are rare in canine SLE; however, subtle behavioral disturbances may go unrecognized in the dog.

Feline SLE

Systemic lupus erythematosus is a rare disease in the cat. Feline SLE involves the musculoskeletal, urinary, cutaneous, and hematopoietic systems with approximately equal frequency.^{28,33,36} Fever is frequently reported in cats. Although the true prevalence of polyarthritis in feline SLE may be underestimated because it tends to be subclinical, articular symptoms seem to be common. Proteinuria or glomerulonephritis also are frequently reported. Cutaneous manifestations include generalized alopecia, erythema, scaling and crusting involving the face, ears, neck, ventrum, and limbs, and crusting of all digital pads. Neurological and behavioral disturbances may be not uncommon in feline SLE and include racing around the house; twitching of the ears, tail, or hind limbs; repeated licking; hyperesthesia along the dorsum; restless crying; disorientation; ataxia; and generalized seizures.^{28,33}

Equine SLE

Equine lupus presents as generalized skin disease (alopecia, dermal ulceration, and crusting), accompanied by an antiglobulin-positive anemia. The disease is remarkable insofar as affected horses may be almost totally hairless. Affected horses may also have glomerulonephritis, synovitis, and lymphadenopathy.¹⁴

DIAGNOSIS

Systemic lupus erythematosus is a diagnosis that must be based on the proper constellation of clinical features and laboratory evidence.

Laboratory Findings

Antinuclear Antibodies

Antinuclear antibodies (see Chapter 140) are found in over 95% of dogs with SLE. The highest titers appear in the most severe clinical cases, and diminish progressively with treatment. The diminution always follows clinical remission, but with a certain time lag. Antinuclear antibodies can be encountered in other diseases, and even in healthy animals (up to 15% of healthy dogs, and around 20% in dogs with various infectious diseases). In the majority of cases their titers are low, but titers higher than 1:1,000 are occasionally found in healthy dogs, notably in German Shepherds.²⁶ The most frequently observed pattern is homogeneous, followed by speckled. The significance of these staining patterns is currently unclear in animals. Although the ANA test is very sensitive, it has a low specificity but is useful as a screening test.

In patients with high clinical suspicion or high ANA titer, additional testing is indicated for evaluation of ANA subtypes (such as histones, Sm, SS-A/Ro, hnRNP G). A number of methods are used to identify antibodies specific for the nuclear components (immunoprecipitation, ELISA, radioimmunoassay, western blotting). Unfortunately, these assays are rarely available outside the research setting. There are two categories of anti-extractable nuclear antigen (ENA) antibodies that, although their sensitivity is low, seem to be particularly interesting because of their high specificity for canine SLE. These categories are anti-Sm antibodies (found in 16% of patients), and anti-hnRNP G (found in 20% of patients).^{5,26}

Other Immunodiagnostic Investigations

Formerly, the lupus erythematosus (LE) cell test was used for diagnosis of SLE. LE cells are neutrophils that have phagocytosed nuclei from dead and dying cells. Their presence may be detected in bone marrow and occasionally in buffy coat preparations. It is usually necessary to produce them *in vitro* using heparinized or clotted blood from patients. There is a high incidence of both false-positive and false-negative results. Because of this, the LE cell test is mostly of historical significance.

A wide variety of autoantibodies can be detected in sera or tissue: rheumatoid factors (seen at low titers in 20% of patients);⁴ antiphospholipid antibodies; Coombs' test to demonstrate anti-RBC antibodies; tests for anti-platelet antibodies (see Chapter 140).

A skin biopsy can help to diagnose SLE with histopathologic evidence of an interface dermatitis or vasculitis. The demonstration of immune deposits at the basement membrane zone (the "lupus band test") by immunofluorescence or immunohistochemistry in both affected and unaffected sites that have been exposed to sun, although not specific, is supportive of the diagnosis of SLE. A renal biopsy is useful in identifying the specific type of glomerulonephritis present.

Circulating immune complex (CIC) level, serum complement function, and concentration of specific complement components also can be measured. Circulating immune complex will decrease and complement levels will rise to normal during clinical remission or following therapy. Reduced levels of complement in canine SLE are less likely to reflect inherited complement deficiency than utilization in immune complex clearance.

Other Laboratory Tests

Laboratory tests to diagnose possible SLE should include: a complete blood count to screen for leukopenia, lymphopenia, anemia, and thrombocytopenia; urinalysis to evaluate for proteinuria, hematuria, casts, or pyuria; kidney and liver serum chemistry tests to monitor kidney disease activity and response to immunosuppressive and anti-inflammatory drugs; and inflammatory markers (e.g. C-reactive protein). The latter may be elevated in any inflammatory condition, including SLE, but level changes could be interesting for monitoring disease activity.

Diagnostic Criteria

Many diagnostic criteria have been proposed for SLE in veterinary medicine, and have been modified from those given for humans. The American College of Rheumatology (ACR) established eleven criteria in 1982 that were updated in 1997 (Table 54.3).^{16,34} These criteria summarize features necessary to diagnose SLE. However, more than diagnostic criteria, they were established for classification and use in scientific research (i.e. inclusion in randomized controlled trials); therefore, patients may have lupus but not meet all of the criteria.

In dogs and cats, the most frequently accepted and simplest diagnostic criteria include the presence of two separate manifestations of autoimmunity, together with significantly elevated titers of serum ANAs.³² For a more stringent diagnosis, it would be recommended to use the 1982 revised ACR criteria that have been proposed and adapted for dogs (Table 54.4).^{7,32} A rigorous approach (Fig. 54.4) is necessary for recognizing this complex disease and assessing particular cases of SLE.

Differential Diagnosis

Systemic lupus erythematosus is a challenging disease whose wide variety of manifestations makes it part of the differential diagnosis for patients with many different presenting complaints (e.g. fever of unknown origin, polyarthritis, IMHA, and nephritis). Infectious and neoplastic diseases must be excluded through imaging and appropriate biological testing, such as serology or polymerase chain reaction (PCR) for vector-borne diseases (e.g. ehrlichiosis, leishmaniasis).

TABLE 54.3 American College of Rheumatology (ACR) Classification Criteria for the Diagnosis of Systemic Lupus Erythematosus (Established in 1982, Updated in 1997)^{a,b}

1. **Serositis:** pleuritis, by convincing history of pleuritic pain, rub heard by physician, or evidence of pleural effusion; or pericarditis documented by electrocardiography, rub heard by physician, or evidence of pericardial effusion
2. **Oral ulcers:** oral and nasopharyngeal ulceration, usually painless, observed by physician
3. **Arthritis:** non-erosive arthritis involving two or more peripheral joints, characterized by swelling, tenderness, or effusion
4. **Photosensitivity:** skin rash as a result of unusual reaction to sunlight, as determined by patient history or physician observation
5. **Hematologic disorder:** hemolytic anemia with reticulocytosis; or leukopenia, $<4.0 \times 10^9/L$ on two or more occasions; or lymphopenia $<1.5 \times 10^9/L$ on two or more occasions; or thrombocytopenia $<100 \times 10^9/L$ in the absence of offending drug
6. **Renal disorder:** persistent proteinuria $>0.5g$ per day, or $>3+$ if quantification is not performed; or cellular casts (may be red blood cell, hemoglobin, granular, tubular, or mixed cellular casts)
7. **Antinuclear antibodies:** an abnormal ANA titer by immunofluorescence or equivalent assay at any time and in the absence of drugs known to be associated with drug-induced lupus
8. **Immunologic disorder:** antibody to dsDNA antigen (anti-dsDNA) in abnormal titer; or presence of antibody to Sm nuclear antigen (anti-Sm); or positive finding of anti-phospholipid antibody based on an abnormal serum level of IgG or IgM anticardiolipin antibodies, a positive test result for lupus anticoagulant using a standard method, or a false-positive serologic test for syphilis that is known to be positive for at least 6 months and is confirmed by negative *Treponema pallidum* immobilization or fluorescent treponemal antibody absorption test
9. **Neurologic disorder:** seizures or psychosis occurring in the absence of offending drugs or known metabolic derangement (e.g. uremia, ketoacidosis, electrolyte imbalance)
10. **Malar rash:** fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds
11. **Discoid rash:** erythematous raised patches with adherent keratotic scaling and follicular plugging; possibly atrophic scarring in older lesions

^aANA, antinuclear antibody; dsDNA, double-stranded DNA.

^bAny combination of 4 or more of 11 criteria, well-documented at any time during a patient's history, makes it likely that the patient has SLE (specificity and sensitivity 95% and 75%, respectively).

TABLE 54.4 Proposed Criteria for the Diagnosis of Canine Systemic Lupus Erythematosus^{a,b}

1. **Arthritis:** non-erosive arthritis involving two or more peripheral joints, characterized mainly by pain during movement (progressive forced flexion-extension), where swelling, or effusion are often not very marked
2. **Renal disorder:** persistent proteinuria in absence of urinary tract infection; or cellular casts (red blood cell, hemoglobin, granular, tubular, or mixed cellular casts)
3. **Cutaneous lesions:** depigmentation, erythema, erosions, ulcerations, crusts and keratotic scaling which selectively affect the areas of thin skin, or skin poorly protected by the hair-coat (in particular, the face: nose pad, forehead, lips and the periocular region)
4. **Oral ulcers:** oral or nasopharyngeal ulceration, usually painless
5. **Hematologic disorder:** hemolytic anemia; or leukopenia, $<3.0 \times 10^9/L$ on two or more occasions; or lymphopenia, $<1.0 \times 10^9/L$ on two or more occasions; or thrombocytopenia, $<100 \times 10^9/L$ in the absence of offending drugs
6. **Serositis:** presence of a non-septic inflammatory cavity effusion (pleuritis or pericarditis)
7. **Neurologic disorders:** seizures or psychosis in the absence of offending drugs or known metabolic disorders (e.g. uremia, ketoacidosis, or electrolyte imbalances)
8. **Idiopathic inflammatory disease of the skeletal or cardiac muscles:** polymyositis or myocarditis
9. **Antinuclear antibody:** an abnormal ANA titer by immunofluorescence or an equivalent assay at any time and in the absence of drugs known to be associated with their formation
10. **Immunologic disorder:** antibody to histone; or antibody to the Sm nuclear antigen; or antibody to the hnRNP G nuclear antigen; or decrease in the CD8 population, $<0.2 \times 10^9/L$; or CD4/CD8 ratio higher than 4.0; or positive finding of anti-phospholipid antibody

^aANA, antinuclear antibody; RNP, ribonucleoprotein

^bAny combination of 4 or more of 10 criteria, well-documented at any time during a patient's history, makes it likely that the dog has SLE.

PROGNOSIS AND TREATMENT

Prognosis

The disease is progressive so that the severity of lesions and the number of organ systems involved increases gradually in untreated cases. Many patients die from

renal failure. The earlier a diagnosis is made, the better the chance in-depth treatment will lead to a cure. Secondary infections are an ever-present risk in animals on immunosuppressive therapy.

Clinical practice must take account of both activity and severity in establishing a prognosis. A dog suffering from slight polyarthritis cannot be compared to one

SLE, but treatment of reported equine cases has been unsuccessful so far.

Levamisole

Levamisole is an anthelmintic drug, also used as an immunoadjuvant that stimulates T cell responses. It probably enhances suppressor cell function. Levamisole has been used with success in canine SLE.^{6,12} Levamisole is administered orally at 5mg/kg every 48 hours (a maximum of 150mg/dog is used) together with prednisone (0.5–1mg/kg twice a day). The prednisone is progressively tapered and discontinued after 2 months, while levamisole is given continuously for 4 months and then stopped. If relapse occurs, levamisole alone is reinstated for a further 4-month period. This regimen is reported to produce long-term remission in over 50% of dogs with SLE. Side effects of long-term levamisole treatment are rare in dogs (neutropenia, aggressiveness, excited behavior).

Other Alternatives

In human SLE, polyarthritis is treated initially with non-steroidal anti-inflammatory drugs. Antimalarial agents (hydroxychloroquine) are used to control skin and joint symptoms and protect against the development of systemic disease. Selective immunotherapy methods are under investigation. The use of CTLA-4-Ig is one of the targeted therapeutic approaches in SLE. Results obtained in the canine SLE experimental model are promising.¹⁰

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Feline Immunodeficiency Virus

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Incidence and Epidemiology
 Pathogenesis
 Clinical Disease
 Hematologic Disorders
 Immune Reaction
 Diagnosis
 Direct Detection of FIV
 Virus isolation
 Polymerase chain reaction

Detection of Antibodies to FIV
 Treatment
 Supportive Treatment
 Antiretroviral Drugs
 Immune Modulators
 Prognosis

Acronyms and Abbreviations

3TC, 2',3'-dideoxy-3'-thiacytidine; AZT, 3'-azido-2',3'-dideoxythymidine; CNS, central nervous system; CTL, cytotoxic T lymphocyte; ELISA, enzyme-linked immunosorbent assay; env, viral envelope; EPO, erythropoietin; FeLV, feline leukemia virus; FIV, feline immunodeficiency virus; gag, group-specific antigen; GM-CSF, granulocyte-macrophage colony-stimulating factor; HIV, human immunodeficiency virus; IFN, interferon; IGF, insulin-like growth factor; MDA, maternal-derived antibody; PCR, polymerase chain reaction; PME, 9-(2-phosphonomethoxyethyl) adenine; pol, polymerase; RBC, red blood cell; RT-PCR, reverse transcriptase PCR; TM, transmembrane; WBC, white blood cell.

Feline immunodeficiency virus (FIV) which was discovered in 1987 in California³⁸ is a retrovirus of the genus *Lentivirus* that is closely related to the human immunodeficiency virus (HIV), sharing a similar structure, life cycle, and pathogenesis.⁵¹ Feline immunodeficiency viruses belong to a large and ancient group; species-specific strains have been isolated from a variety of non-domestic Felidae, including the puma, lion, leopard, and pallas cat.^{4,6,9,35} As with all lentiviruses, FIV is a complex retrovirus, containing accessory genes in addition to *gag*, *pol* and *env*. The FIV *gag* gene encodes, among others, the capsid protein p24; the *pol* gene encodes the protease, integrase and reverse transcriptase and additional enzymes important to the virulence of FIV. Both *gag* and *pol* are relatively conserved between strains. The *env* gene encodes the viral surface glycoprotein (gp120) and the transmembrane protein (gp41), with the gp120 carrying the major determinants of viral diversity amongst isolates.³⁵ Five genetically distinct subtypes or clades (designated A to E) have been defined, that exhibit considerable sequence diver-

sity (up to 26%) within the *env* genes.⁵² The majority of viruses identified so far belong to either subtype A or B. Although multiple subtypes have been documented in cats from the same continent, geographic clustering of subtypes is evident (Fig. 55.1).

INCIDENCE AND EPIDEMIOLOGY

Feline immunodeficiency virus is endemic in domestic cat populations worldwide; the seroprevalence is highly variable between regions, with estimates of 1–14% in cats with no clinical signs and >20% in sick cats (Table 55.1). Sick adult cats, male cats, and free-roaming cats are most likely to be infected. The major route of natural transmission is believed to be via inoculation of saliva during fighting. Vertical transmission and transmission between socially well adapted cats appear to be relatively infrequent but have been described.¹ The majority of natural FIV infections are acquired by biting, presumably through the inoculation of virus, or virus-



FIGURE 55.1 Global distribution of FIV subtypes or clades. (Reproduced with permission from the European Advisory Board on Cat Diseases [ABCD]; <http://www.abcd-vets.org>.)

TABLE 55.1 Prevalence of FIV Infection Segregated by Geographic Area and Cat Population

Geographic Area	Prevalence	Cat Population
Australia	6.5% (<i>n</i> = 200)	Healthy cats
	20.8% (<i>n</i> = 711)	Sick cats
Canada	7.6% (<i>n</i> = 607)	
Czech Republic	5.8% (<i>n</i> = 727)	
France	22.1% (<i>n</i> = 208)	Sick cats
Germany	5.4% (<i>n</i> = 1,911)	Random cats
Great Britain	12.8% (<i>n</i> = 431)	Sick cats
	27% (<i>n</i> = 90)	Feral cats
Netherlands	3% (<i>n</i> = 98)	Sick cats
Norway	10.1%	Sick cats
	5.9% (<i>n</i> = 224)	Healthy cats
Switzerland	3.7% (<i>n</i> = 775)	Sick cats
	2.8% (<i>n</i> = 178)	Healthy cats
USA, California	14.4% (<i>n</i> = 1,612)	
USA, Raleigh	2.3% (<i>n</i> = 733)	Unowned cats
USA, Florida	4.3% (<i>n</i> = 1,043)	Unowned cats
USA	2.5%	18,000 cats

infected cells, from the saliva of persistently infected cats. Transmission from mother to kittens may occur but only a proportion of the offspring become persistently infected. The proportion of kittens infected by their queens depends on the viral load of the queen during pregnancy and birth. For example, if the queen became infected at the time of mating (being bitten by an FIV infected tom cat), the viral load may be high at birth and up to 70% of the kittens may become infected at birth or via milk.³⁶ In contrast, if the queen had been

infected before conception and was clinically healthy during gestation, the viral load of the queen would be low and the kittens would generally not become infected.² Oronasal or venereal transmission have not been documented under field conditions. However, under experimental conditions, cats can be infected by inoculation of virus into the nose, mouth, vagina, and rectum, and virus can be recovered from semen following natural or experimental infection.

PATHOGENESIS

The major targets for FIV infection are activated CD4+ T cells.²² These cells typically function as T-helper cells which have a central role in immune function, facilitating the development of humoral and cell-mediated immunity. The primary receptor to which gp 120 binds, is the CD134 molecule.⁵⁰ After binding to CD134, a conformational change occurs in gp120 that gives rise to binding between another domain on the gp120 molecule and the viral co-receptor, CXCR4.⁵⁸ These steps mediate fusion of viral and cell membranes and subsequent viral entry. The reverse transcriptase synthesises a DNA strand complementary to the viral RNA that is transported to the nucleus where its insertion into the host DNA in the form of the so-called provirus is mediated by the integrase enzyme. As reverse transcriptase lacks a proofreading function, its fidelity is low and, as a consequence, FIV has a tendency to mutate. This, together with the development of the immune response, is the basis for genetic diversity, resulting in the generation of variants and immune evasion. Latent infection

is characterized by the presence of inactive provirus and a lack of synthesis of new progeny virions. As no viral antigens are exposed on the surface of latently infected cells, latent infection is not recognized by the immune system. Consequently, latently infected cells act as a reservoir of infection that may not be cleared by either a functioning immune system or vaccination.

In the first days after infection, FIV replicates in dendritic cells, macrophages, and CD4+ T lymphocytes. Within 1–2 weeks, a low level viremia develops.³⁴ The viral RNA content in plasma and provirus present in blood mononuclear cells increases with time, with highest loads found between 8 and 12 weeks post-infection.⁷ This so-called “acute phase” of infection is overcome by a functioning immune system, giving rise to a long asymptomatic phase during which viral loads are stable at low levels. As infection progresses, the number of CD4+ T cells gradually decreases, eventually resulting in a decreased CD4:CD8 T cell ratio and an immune deficiency that is found in most infected cats (Fig. 55.2).⁵⁴ The loss of CD4+ cells is associated with apoptosis.⁵ Immunodeficiency together with very high viral loads is accompanied by the clinical signs of AIDS and death.³⁰

CLINICAL DISEASE

During the acute phase of the infection, mild to moderate clinical signs include lymphadenopathy, pyrexia, anorexia, and lethargy. While pyrexia, anorexia, and

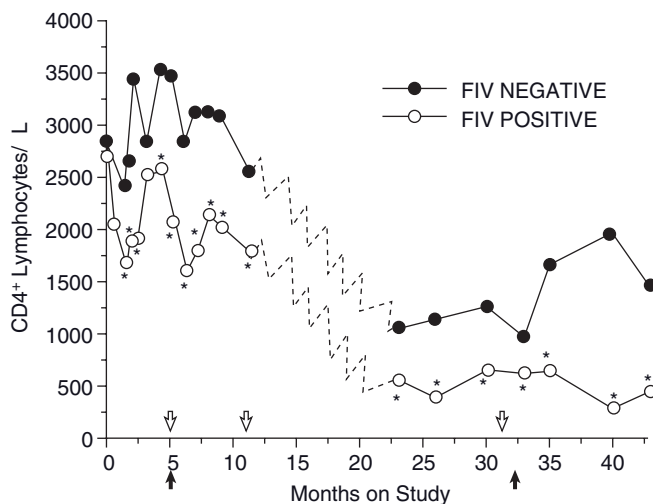


FIGURE 55.2 Decline in CD4+ lymphocyte counts in FIV-infected and control cats. Break marks in the data lines signify a separation into two different groups of cats, those with short-term infections on the left and those with long-term infections on the right. Statistically significant differences ($p < 0.05$) between FIV infected and control cats are indicated by asterisks. (Reproduced from Torten M, Franchini M, Barlough JE, et al. Progressive immune dysfunction in cats experimentally infected with feline immunodeficiency virus. *J Virol* 1991;65:2225–2230, with permission.)

lethargy usually resolve rapidly, lymphadenopathy may persist over many weeks or months. Lymphadenopathy is caused by increased number and size of active germinal centers in lymph nodes. A decrease in plasma viral load marks the beginning of the so-called “asymptomatic” phase that can last for many years, or may be life-long. It is assumed that during the asymptomatic phase, viral replication is controlled by the immune system. As with the progression of HIV infection to AIDS, the course of FIV infection has been categorized into different phases. Progression to immunosuppression depends on the virulence of the infecting strain and age of the cat when infected.³⁹ Immunosuppression progresses faster in young cats than in older cats. If infection was acquired at an older age, progression to immunosuppression may not be seen until very old age or never.³⁰ Immunosuppression frequently manifests as chronic diseases such as persistent stomatitis/gingivitis, chronic rhinitis, skin disease, lymphadenopathy, and weight loss. Infected cats are prone to secondary or opportunistic infections and neoplasia. B cell lymphomas, myeloproliferative disease, and squamous cell carcinoma have been reported in cats with FIV infection. Many concurrent viral, bacterial, fungal, and protozoal infections have been reported in FIV-positive cats, although some studies have found no significant association between FIV infection and pathogens such as *Cryptococcus*, *Cryptosporidium*, *Mycoplasma haemofelis*/*M. haemominutum*.

Chronic diarrhea is a frequent clinical sign associated with FIV infection and can result in severe weight loss. In most cases this cannot be attributed to secondary infection with opportunist organisms and it has been argued that FIV may have a direct effect on the gut.³⁷ Feline immunodeficiency virus was found to also infect the central nervous system (CNS).¹³ Occasionally, FIV infection leads to abnormal behaviour, probably caused by activation of innate immunoreactivity.^{28,44} Disrupted reproduction has been reported in FIV-infected cats when placental and fetal tissues are affected.⁵⁶ Also, an association between FIV infection and glomerular and tubulo-interstitial lesions combined with proteinuria has been documented.⁴³

HEMATOLOGIC DISORDERS

During the acute phase of FIV infection, lymphopenia and severe neutropenia have been described in experimental infections.¹⁴ However, lymphopenia and neutropenia are not consistently found, probably reflecting differences among the FIV strains and differences in infectious doses used. The most frequent hematologic abnormalities in naturally-infected clinically sick cats include anemia, neutropenia, lymphopenia and monocytosis.^{26,30,49} However, during the asymptomatic phase, the number of granulocyte/macrophage progenitors (CFU-GM) and erythroid progenitors (CFU-E and BFU-E, respectively) was found to be essentially unchanged compared to those of uninfected control

cats. The same authors found that sera from FIV-infected cats supported progenitor cell growth *in vitro* as well as normal cat sera. These results suggest that progenitor cells of RBCs and granulocytes/macrophages are not major targets of FIV infection.

IMMUNE REACTION

Soon after the discovery of FIV it was shown that antibodies to a laboratory-adapted FIV strain can confer passive protection against FIV infection.^{25,45,61} After infection, cats readily develop high titers of antibodies to the different FIV components. These can be detected by immunofluorescence assays, enzyme-linked immunosorbent assay (ELISA), or immunochromatography tests.^{33,38} Antibody specificity is usually determined by Western blots or radioimmunoprecipitation.^{17,32} Anti-FIV antibodies are detectable from 2 to 4 weeks post-infection, or later if cats are exposed to low doses of virus.²⁷ Antibodies to gp41 appear within a few weeks after infection; those recognizing Gag and gp120 appear somewhat later.⁸ Virus-neutralizing antibodies develop a few weeks after infection. However, cats cannot clear FIV infection, even if high titers of virus neutralizing antibodies are present.¹⁹ As well as antibodies against FIV, cytotoxic T cells (CTLs) specific for FIV are important for immunity against FIV. However, it has not been fully elucidated to what degree antibodies and CTLs contribute to the control of infection. Cytotoxic T cells seem to have an important role: protection from experimental infection in cats vaccinated with formaldehyde-fixed FIV was found to be associated with the induction of CTLs specific for Gag and Env proteins.³⁴ FIV-specific CD8+ T cells were shown to appear as early as 2 weeks after infection. A population of CD8+ T cells termed CD8^{low} has been observed in early FIV infection with some isolates;⁵⁷ these cells act as a marker of immune activation by more virulent strains of FIV and may contribute functionally to the non-cytolytic activity against FIV mediated by CD8+ T cells.²¹

The gradual loss of CD4+ T cells during FIV infection is usually not used to stage the level of immune dysfunction as the assays are complicated and usually pre-infection cell counts are not available.

DIAGNOSIS

FIV infection can be diagnosed directly by detecting the virus and indirectly by demonstrating the presence of antibodies.

Direct Detection of FIV

Virus Isolation

Isolation of FIV in cell culture was the first method used to demonstrate FIV infection in infected cats.³⁸ Blood lymphocytes are prepared from fresh samples of heparinized blood and are co-cultivated with primary

feline T cells for up to 4 weeks. The presence of virus in culture supernatants is confirmed by measuring the levels of viral core proteins in the culture fluids⁵³ or by quantitation of provirus or FIV RNA using real-time polymerase chain reaction (PCR) or RT-PCR.²⁹ Virus isolation in culture is laborious and not used routinely.

Polymerase Chain Reaction

Polymerase chain reaction-based assays that detect presence of viral RNA or proviral DNA were introduced in 1992.⁴⁷ The advent of real-time PCR allowed also the quantitation of FIV-DNA and RNA.²⁹ PCR-based FIV detection is very sensitive; however, some isolates are not always detected as variations may occur in the DNA or RNA sequences where primers bind. Variation of FIV sequences may also explain discrepant results when identical samples are sent to different laboratories.¹⁰ Discrepant results (seropositive, PCR negative) also can be explained by the presence of an FIV subtype not recognized by the PCR, rather than by the absence of FIV infection. Seropositive results in PCR-negative cats also can be explained by vaccination. In addition, discrepant results (seronegative, PCR positive) have been described in cats living in close contact with FIV-infected seropositive cats.¹¹ These cats have become infected at low levels and will eventually seroconvert once viral loads have increased sufficiently to induce antibody production.

Detection of Antibodies to FIV

During FIV infection, cats develop antibodies to all components of FIV.^{17,27,32} Antibodies to the transmembrane (TM) protein gp 41 appear first, thereafter antibodies to the Gag and envelope glycoprotein gp 120.⁸ For routine detection of antibodies to FIV, purified whole FIV, FIV-infected cells and purified proteins produced by recombinant DNA technology have been described.^{33,53} Serological tests used in diagnostic laboratories are mostly based on a combination of p24 and TM components while immunochromatography tests usually contain synthetically produced parts of TM. Western blots are considered the "gold standard" for FIV serology and are used to confirm inconclusive results.³² ELISA and immunochromatography tests are widely used under practice conditions but the diagnostic specificity of these tests is usually below 100% which means that some results are false positive. This is especially important in populations with low prevalence, such as in many countries in central Europe. If the FIV prevalence is 1% and the diagnostic specificity is 99%, two cats will test positive per hundred cats (i.e. the predictive value for a positive is only 50%). Therefore, any positive result in a cat without the typical history and clinical signs (e.g. young, healthy, indoor, pure bred cats) must be confirmed (e.g. by Western blot). A positive result in a cat from a high-risk group (e.g. a free roaming, older male with scratch wounds) is likely to be a true positive as the frequency of true positives will exceed the frequency of false positives in this

population. In contrast, negative results in low prevalence populations are generally very accurate, with the following exceptions. False negative results may be obtained early in infection, when cats are provirus positive but remain seronegative for several weeks to months. In addition, false negative results may be obtained in the terminal stages of disease due to immunosuppression and when high viral titres may lead to sequestration of anti-FIV antibodies in virus-antibody complexes.

Kittens born to FIV-infected queens may test seropositive as a result of passively acquired maternal antibodies (MDA). In such cases, kittens should be retested at approximately 16 weeks of age, by which time levels of MDA will have declined to undetectable levels. However, in rare cases antibodies may persist for up to 6 months. Therefore, a kitten testing seropositive at 16 weeks old should be retested 2 months later. If it is still positive at 6 months it is infected. If an earlier result is required, PCR may be used; however, it is important that the queen is tested in parallel to ensure that the PCR can detect the strain of virus causing the seropositive reaction.

Vaccination of cats against FIV infection with an inactivated, whole virus vaccine results in rapid and persistent production of antibodies that are indistinguishable from those used for diagnosis of FIV infection. This vaccine is available in the USA, Australia, New Zealand, and Japan but is not licensed in Europe. Vaccinated cats may be imported from a country where the vaccine is used and will test positive for antibodies to FIV. In such cases a positive test cannot distinguish between infection and vaccination.⁴⁶ An ELISA procedure has been described that may permit distinction between vaccination- and infection-induced antibodies.³¹

TREATMENT

Supportive Treatment

Symptomatic treatment of infected cats should be directed at the clinical signs that arise from secondary infections. During the early phase of FIV infection, cats usually respond as well as FIV-negative cats to appropriate medications, although a longer or more aggressive course of therapy (e.g. antibiotics) may be required. There are reports of clinical benefits using corticosteroids and other immune suppressive drugs in FIV-infected cats with chronic stomatitis, but their use is controversial because of potential side effects. Griseofulvin has been shown to cause bone marrow suppression in FIV-infected cats and therefore should not be used.⁴⁸

Antiretroviral Drugs

There is no treatment of FIV infection that leads to complete clearance of the virus. However, as in HIV infection, there are several drugs with proven antiret-

roviral activity. As HIV and FIV share many characteristics, the mode of action and the efficacies of many antiretrovirals are similar for HIV and FIV.

Azidothymidine (AZT, 3'-azido-2',3'-dideoxythymidine) is a nucleoside analog reverse transcriptase inhibitor that inhibits the reverse transcriptase of HIV and FIV and was shown to lead to significant improvement of clinical signs in FIV infected cats.²³ AZT can be given by injection or orally. Side effects of AZT (nonregenerative anemia) are seen in some cats, especially when the drug is used at higher doses. Other nucleoside analogs with a similar mode of action to AZT are used in HIV infection and are also efficacious in FIV infection. Stampidine (2',3'-didehydro-3'-deoxy-5'-thymidylyl-l-alanine methyl ester) was shown to inhibit FIV replication in vitro and to be well tolerated by cats.⁵⁵

Lamivudine (2',3'-dideoxy-3'-thiacytidine, 3TC) was evaluated in vitro and in vivo in experimental infection and was found to inhibit FIV. The combination of 3TC and AZT was highly effective for prophylaxis but only marginally effective in chronically FIV-infected cats.³ The acyclic purine nucleoside analogue (PMEA) was shown to efficiently inhibit FIV replication and to improve, in a dose dependent manner, clinical signs of FIV infection.¹⁶ However, PMEA exhibited strong side effects, especially at higher doses, that precluded its use in privately owned cats with FIV infection. Enfuvirtide or T20 is a synthetic polypeptide with a strong similarity to the HIV TM protein. Enfuvirtide binds strongly to gp41 and thereby prevents the fusion of the virus with the cell; hence it is a fusion inhibitor. Similarly, an analogue to the FIV transmembrane protein was shown to inhibit FIV infection in vitro and to lead to a reduced viral load in vivo.¹⁵ In spite of the fact that this fusion inhibitor is well tolerated by the cats, its use is hampered as it has a short half-life and has to be injected subcutaneously. Bicyclams, molecules developed in the late 1980s as anti-HIV drugs, were also found to inhibit FIV.¹⁸ They interfere with the CXCR4 molecule, the primary receptor for FIV.^{57,58} So far, no studies have been published on the use of bicyclams in naturally FIV infected cats.

Immune Modulators

Use of recombinant human granulocyte-macrophage colony-stimulating factor, (rHuGM-CSF) resulted in an increase in total white blood cell (WBC) counts, attributed to an increase in either neutrophils, lymphocytes, eosinophils, monocytes, or combinations thereof. In addition, blood mononuclear cell-associated FIV load in some of the treated cats increased by 1–2 logs.³ Therefore, use of rHuGM-CSF in FIV infection is currently not recommended. When human recombinant granulocyte colony-stimulating factor (rHuG-CSF) was evaluated, significant increases in both neutrophils and the myeloid to erythroid ratio in bone marrow were observed without increased FIV expression.⁴² However, 14–21 days after initiation of treatment, rHuG-CSF gave rise to the synthesis of anti-rHuG-CSF antibodies in some

of the cats.⁴² Recombinant human erythropoietin (rHuEPO) led to a gradual increase in RBC and WBC counts in FIV-infected cats and because no increase of virus load was observed, the authors concluded that rhEPO can be used safely in FIV-infected cats.³ Insulin-like growth factor-1 (IGF-1) is available as recombinant human product (rHuIGF-1).⁵⁹ Besides other actions, it has the capacity to induce thymic growth and to stimulate T cell function. In cats experimentally infected with FIV, the use of rHuIGF-1 resulted in increased thymus size and thymic cortical regeneration, leading to an increase in T cell numbers in the blood. Therefore, rHuIGF-1 could be considered as a supportive treatment in younger cats with FIV infection. Several cytokines have been evaluated for their capacity to inhibit FIV infection. Recombinant human interferon alpha (rHuIFN α) which mainly exerts an antiviral effect was administered orally in low doses (10 IU/kg per day) to cats naturally infected by FIV. Interestingly, the low-dose human IFN α treatment significantly prolonged the survival of the FIV-infected cats and improved their clinical signs compared to cats in the placebo group.⁴¹ In 1986, Yamamoto and colleagues described a cell line producing a feline interferon that resembled feline IFN α ;⁶⁰ later this was designated IFN ω . Recombinant IFN ω was shown to exert a strong anti-FIV effect in vitro and in vivo.⁶⁰ In a double blind study, treatment resulted in lower mortality and improvement of abnormal hematologic parameters.¹² However, there was no difference in the survival rates of treated and untreated cats.

PROGNOSIS

Disease progression in FIV infection is similar to that of HIV infection and under natural conditions, development of clinical signs may take many months to years. The mean survival time in untreated FIV-infected cats after diagnosis was reported to be slightly more than 2 years.²⁰ After the acute phase of infection, that develops a few weeks after infection, an asymptomatic phase follows which is paralleled by low but clearly detectable viral loads. Many months to years later, viral loads increase again and the CD4+ cell numbers and the CD4+:CD8+ ratio decrease.⁵⁴ Clinical signs of immune suppression and secondary infections usually manifest themselves when the number of CD4+ cells in blood falls below approximately 200 cells/ μ L.²⁴ The kinetics of disease progression is directly dependent on the initial FIV load in plasma and therefore mainly dependent on the virulence of the strain. In addition, the clinical course of FIV infection depends on the sex, age, roaming habits, and the immune status of the affected cat. Co-infections, especially with feline leukemia virus, lead to an accelerated disease progression.⁴⁰

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T Cell, Immunoglobulin, and Complement Immunodeficiency Disorders

PETER J. FELSBURG

Immunodeficiency Disease
 Diagnosis of an Immunodeficiency
 Deficiencies Involving the B Cell (Humoral) Immune System
 Equine Agammaglobulinemia
 Selective IgA Deficiency
 IgA deficiency in humans
 IgA deficiency in dogs
 Equine Selective IgM Deficiency
 Transient Hypogammaglobulinemia of Infancy in Dogs
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Deficiencies Involving the T Cell (Cell-Mediated) Immune System
 Hypotrichosis with Thymic Aplasia (Nude Kittens)
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 Deficiencies of the Complement System
 C3 Deficiency

Acronyms and Abbreviations

Btk gene, Bruton tyrosine kinase gene; CVID, common variable immunodeficiency; HLA, human leukocyte antigen; IgA, immunoglobulin A; IgAD, IgA deficiency; IgG, immunoglobulin G; IgM, immunoglobulin M; SC, secretory component; sIgA, secretory IgA; XLA, X-linked agammaglobulinemia; *whm*(winged-helix-nude) gene.

IMMUNODEFICIENCY DISEASE

Immunodeficiency diseases, characterized clinically by an increased susceptibility to infection, are a diverse group of diseases that result from abnormalities in one or more components of the immune system. Immunodeficiency diseases can be broadly divided into two main categories: primary, or genetic, immunodeficiencies and secondary, or acquired, immunodeficiencies. Primary immunodeficiencies are diseases in which the animal is born with a genetic defect involving the immune system and clinical disease is a direct consequence of the hereditary defect. Because the primary immunodeficiency diseases are genetic, the majority of affected patients are neonates or young animals. Acquired immunodeficiencies are diseases in which the animal is born with an intact immune system, but as a result of some underlying disease process, their immune system becomes transiently or permanently impaired.

Animals that have immunodeficiencies generally suffer from recurrent or chronic infections. Some of the more frequent conditions associated with immunodeficiency diseases are respiratory infections, otitis, dermatitis, and pyoderma, diarrhea, growth retardation, adverse reactions to modified-live vaccines, and infection with usually nonpathogenic organisms (opportunistic infections). Although the possibility of an immunodeficiency should be considered in any animal that has too many infections, primary immunodeficiency diseases are relatively infrequent, so it is important to consider other causes that may lead to infection. Most of these conditions can be identified after a careful history and physical examination and can be confirmed by appropriate laboratory tests. When there is no apparent explanation for the recurrent infections, a primary immunodeficiency should be considered.

Several important primary immunodeficiency diseases are not discussed in this chapter, but can be found in Chapters 42 and 57. These diseases include canine

X-linked severe combined immunodeficiency, severe combined immunodeficiency in Arabian foals, severe combined immunodeficiency in Jack Russell terriers, and leukocyte adhesion deficiency in dogs and cattle.

DIAGNOSIS OF AN IMMUNODEFICIENCY

The severity of the defect and which part of the immune system is affected influences the type of infection involved and the clinical signs. Defects in the B cell (humoral immune) system usually result in increased susceptibility to bacterial infections. Animals with T cell (cell-mediated immune) deficiencies usually have an increased susceptibility to intracellular microorganisms such as fungal, protozoal, and viral infections. Note that since a humoral immune response is highly dependent on the T cell system, certain T cell deficiencies may also present as humoral immune deficiencies. Disorders of the phagocytic system are usually associated with skin infections or systemic infections with pyogenic microorganisms. Complement deficiencies are usually associated with recurrent infections with pyogenic microorganisms.

Although the clinical history and findings may be highly suggestive of an immunodeficiency, the diagnosis must be established by appropriate clinical immunologic testing. Many tests of immune function are available; however, the clinical immunologic tests listed below are commonly used to initially determine the competence of the various components of the immune system. These basic tests are becoming more available to the veterinary practitioner. Many of these tests are explained in detail in other chapters of this book (see Section X). Once an immunodeficiency has been established, other more sophisticated tests can be performed to localize the defect more precisely.

1. B cell system
 - Quantitation of serum immunoglobulin A (IgA), immunoglobulin G (IgG), and immunoglobulin M (IgM)
 - Phenotypic evaluation of B cells by flow cytometry.
2. T cell system
 - Lymphocyte transformation test
 - Phenotypic evaluation of T cells by flow cytometry.
3. Phagocytic system (see Chapter 141)
 - Bactericidal assay.
4. Complement system
 - Quantitation of serum C3.

The most practical test for evaluating the humoral immune system is to quantitate the serum concentrations of IgG, IgM, and IgA. Since the only published normal values are from normal adult animals, it is important not to overemphasize the significance of low immunoglobulin concentrations in young animals. Serum immunoglobulin concentrations are greatly influenced by age. For example, in dogs, IgM concentra-

tions reach normal adult values within several months but IgG concentrations do not reach normal adult values until approximately 10–12 months of age and IgA concentrations until approximately 15–18 months of age. It is imperative to compare the immunoglobulin concentrations of young animals with age-matched healthy animals. If the immunoglobulin concentrations are less than 95% confidence limits for age-matched healthy animals, a diagnosis of a deficiency in one or more of the immunoglobulin classes (antibody deficiency) may be made. The lymphocyte transformation (blastogenesis) test is the simplest and most widely accepted *in vitro* test to evaluate the competence of the T cell system. This test evaluates the capacity of T cells to proliferate after stimulation. Animals with T cell deficiencies have an absent or a significantly reduced response to stimulation. If an abnormal lymphocyte transformation response is found in an animal, it is important to test the animal on at least two occasions to eliminate the possibility of transient T cell suppression from concurrent viral infection.

Because the main function of the phagocytic (neutrophil) system is to phagocytize and eliminate microorganisms from the body, neutrophil function is most frequently evaluated by bactericidal assays. This *in vitro* test measures the capacity of neutrophils to kill bacteria. It is important to test a healthy animal at the same time as the patient to control for test variables.

Several tests of the complement system exist. However, since the only documented complement deficiency is a C3 deficiency, quantitation of serum C3 concentrations is sufficient.

DISEASES INVOLVING THE B CELL (HUMORAL) IMMUNE SYSTEM

Equine Agammaglobulinemia

Agammaglobulinemia is a rare immunodeficiency that has been reported in Thoroughbreds and Standardbreds.^{3,11} It has only been reported in males, suggesting an X-linked mode of inheritance with many similarities to X-linked agammaglobulinemia (XLA) in humans. XLA in humans is caused by a mutation in the Bruton tyrosine kinase (Btk) gene that is critical for normal B cell development.

Affected horses suffer from recurrent bacterial infections, such as pneumonia, enteritis, laminitis and arthritis. Clinical signs begin between 2 and 6 months of age with most affected horses dying before 2 years of age from generalized infections. The two major laboratory findings are the lack of peripheral B cells and low or absent IgA, IgG, and IgM. Specific antibody responses also are severely depressed. T cell function in these horses is normal.

Treatment is symptomatic including antibiotic therapy. Although administration of plasma may temporarily control infections, the long-term prognosis is poor.

Selective IgA Deficiency

A deficiency of IgA results in failure to mount a local immune response to bacteria and viruses, predisposing the individual to infections. IgA-deficient individuals also have an increased absorption of antigens, predisposing them to potential development of allergies and autoimmune disease. IgA deficiency has only been documented in humans and dogs.

IgA Deficiency in Humans

IgA deficiency (IgAD) is the most frequent primary immunodeficiency in humans.^{5,8,19} The prevalence, based upon screening large populations of healthy adult blood donors, is approximately 1/600 in Western populations. IgAD is actually a heterogeneous group of diseases consisting of two main types: severe IgAD defined as undetectable IgA, and partial IgAD defined as low IgA. A further complication of IgAD, particularly in pediatric patients, is that it may be transient, with IgA levels normalizing by adulthood. This phenomenon is observed in approximately 8% of severe IgAD and 50% of partial IgAD pediatric patients.¹⁸

Although individuals with IgAD are frequently asymptomatic, reduced levels of IgA predispose to a variety of diseases, including upper respiratory infections, skin infections, neurologic problems, allergies, and autoimmune disease. In fact, symptomatic IgAD accounts for 10–15% of all cases of clinically important primary immunodeficiencies. Approximately 50% of IgAD patients present with recurrent infections, particularly of the upper respiratory tract. Other clinical conditions include chronic skin infections, atopic disease, nonspecific dermatitis, eczema, rheumatoid arthritis, and systemic lupus erythematosus.

Although the familial aggregation of IgAD is highly suggestive of a genetic basis for this condition, the mode of inheritance is unknown. There are associations with certain human leukocyte antigen (HLA) types.

IgA Deficiency in Dogs

The initial descriptions of IgAD in the dog were in the Beagle and Shar-pei.^{12,20} The most frequent clinical problems in dogs with IgAD include recurrent upper respiratory infections due to *Bordetella bronchiseptica* and canine parainfluenza virus, otitis, staphylococcal dermatitis, and atopic dermatitis. The infections associated with IgAD in the dog begin in the first few months of life but are usually not life threatening. Several dogs have experienced convulsive episodes. During a 5 year study of dogs with chronic skin disease, 40 were identified with IgAD.⁶ Breeds represented in this study included Shar-peis, German Shepherds, Cocker spaniels, Doberman pinschers, miniature Schnauzers, miniature pinchers, Akitas, and isolated cases in Yorkshire terriers, Welsh corgi, Newfoundland, West Highland White terrier, Keeshound, Irish setter, Wheaten terrier, and Old English sheepdog. The two major clinical presentations were atopy with secondary staphylococcal

pyoderma (14 dogs) and chronic or recurrent staphylococcal pyoderma (12 dogs).

In a one year prospective study of Beagle puppies diagnosed with IgAD, the incidence of upper respiratory disease was 2.4 times greater in the IgAD dogs than in age-matched dogs with normal IgA concentrations. In addition, the IgAD dogs were three times more likely to be infected with *Giardia* than were dogs with normal IgA concentrations.¹³

Screening of large populations of adult dogs has demonstrated that IgAD is present in clinically normal adult dogs and can take the form of either severe or partial IgAD. Determination of IgA concentrations in 829 clinically normal adult Beagles revealed the presence of IgAD in approximately 9% of the dogs, 1% with severe IgAD and 8% with partial IgAD.¹³ IgAD diagnosed in these healthy adult dogs appears to be persistent, similar to the observation in humans. A 2 year study of five severe IgAD and 15 partial IgAD healthy adult Beagles showed that all five of the dogs with severe IgAD remained severely IgA-deficient and 12 of 15 dogs with partial IgAD remained partially IgA-deficient. Six of these dogs developed nonspecific dermatitis during the study period, with 50% of these dogs having positive skin tests for several environmental allergens. Thirty-two percent of these dogs developed rheumatoid factor in the absence of overt clinical rheumatoid arthritis. Anti-IgA antibodies were found in 30% of the IgAD dogs. Partial IgAD has been reported to occur in up to 75% of clinically normal adult Shar-pei dogs,²⁰ and has been reported in German Shepherd dogs.^{28,10}

Although the mode of inheritance of IgAD in dogs remains unknown, epidemiologic studies have shown that puppies born to clinically healthy IgAD mothers are at much higher risk of developing upper respiratory infections than puppies born to mothers with normal IgA concentrations.²⁶ An experimental breeding of an IgAD dam and IgAD sire resulted in a litter of five puppies. At 3 months of age, four of five puppies had undetectable IgA; at 1 year of age, one dog still had severe IgAD and two dogs had partial IgAD. All three IgAD dogs experienced recurrent upper respiratory infections during the one-year study.¹³ The severe IgAD dog also experienced chronic dermatitis and several episodes of seizures.

The diagnosis of IgAD, both in humans and dogs, is based upon the quantitation of serum IgA. An important consideration in the diagnosis of IgAD is the age of the dog.¹⁶ Levels of serum IgA are low in all puppies. By 3–4 months of age, serum IgA levels have increased to levels at which normal and IgAD dogs can be differentiated. Unlike IgM and IgG, serum IgA concentrations do not reach normal adult levels until 12–18 months of age. Therefore, it is imperative to compare a young, potentially IgAD dog with values for age-matched normal dogs. Another important consideration is the transient IgAD observed in some pediatric patients. Approximately 20% of dogs diagnosed with IgAD before 1 year of age will revert to normal IgA levels between 12 and 18 months of age; however,

approximately 90% of dogs diagnosed with IgAD after 1 year of age will remain IgAD.

Treatment of IgAD patients is limited to symptomatic treatment of the various infections, allergies, and/or autoimmune diseases. No specific immunotherapy exists for the treatment of IgAD. Immune globulin is contraindicated because many IgAD patients may possess or produce anti-IgA antibodies that could result in anaphylactic reactions. In addition, it would not replace sIgA that is the cause of IgAD.

Equine Selective IgM Deficiency

Traits that characterize selective IgM deficiency are reduced or absent serum IgM with normal or elevated serum concentrations of other immunoglobulin classes.^{21,27} Although it occurs in different breeds of horses, it is most frequent in Arabians and Quarter Horses. The mode of inheritance is unknown. Two forms of the disease have been described. The most frequent form occurs in foals that develop severe pneumonia, arthritis, enteritis, and possibly septicemia. The bacterial infections are frequently caused by *Klebsiella* sp. Most of these foals die before 10 months of age. The second form also occurs in foals, but these foals may respond temporarily to antibiotic therapy resulting in recurrent infections and poor development. These foals usually die between 1 and 2 years of age.

The only significant laboratory abnormality is an absent or low serum IgM and is the basis for the diagnosis of this disease. T cells in horses with selective IgM deficiency have normal T cell function. In contrast to equine agammaglobulinemia, the proportion and absolute numbers of both B cells and T cells are normal. Other than supportive care and antibiotic therapy, there is no effective treatment for selective IgM deficiency.

Transient Hypogammaglobulinemia of Infancy in Dogs

Transient hypogammaglobulinemia of infancy is a self-limited immunoglobulin deficiency resulting from a delayed onset of IgG and IgA production by the neonate or young puppy.¹⁴ Affected puppies are clinically normal during the time they possess maternal antibody. However, when the maternal antibody disappears, they have increased susceptibility to infection, primarily chronic or recurrent bacterial infections of the respiratory tract. Spontaneous recovery occurs between 5 and 7 months of age when the dog's own humoral immune system begins to produce sufficient immunoglobulin. Symptomatic treatment may be needed during this period.

The only significant laboratory finding is markedly reduced concentrations of serum immunoglobulin after the disappearance of maternal antibody, at around 2 months of age, that persists until 5–7 months of age. It is essential to monitor the immunoglobulin concentrations of puppies diagnosed as immunoglobulin deficient to determine if the defect is permanent or transient.

Immunodeficiency Syndrome in Shar-pei Dogs

A late-onset immunodeficiency that appears to be similar to common variable immunodeficiency in humans has been described in Shar-pei dogs.²² Human common variable immunodeficiency (CVID) is a late-onset hypogammaglobulinemia with onset of symptoms between 20 and 30 years of age on average.^{2,9} Common variable immunodeficiency patients suffer from recurrent upper respiratory and gastrointestinal infections and have an increased incidence of autoimmune disorders and malignancies, primarily malignant lymphoma and gastrointestinal adenocarcinomas. The major immunologic finding is low concentrations of two or all three of the immunoglobulin isotypes.

Affected dogs have normal proportions of peripheral B and T cells; however, they do demonstrate abnormalities in B and T cell function. The major laboratory findings are low serum concentrations of one or more of the serum immunoglobulins. Most affected dogs had low serum concentrations of IgM and IgA, and some dogs also had low levels of serum IgG. Approximately half of affected dogs had depressed T cell proliferative responses following mitogenic stimulation that correlated, like human CVID patients, with decreased serum concentrations of IgG.

The disease in Shar-peis is characterized by intermittent fever and recurrent infection of skin, respiratory system, and gastrointestinal system, including ulcerative colitis. The mean age at clinical onset is 3 years. In several reported cases, death occurred as the result of intestinal adenocarcinoma and malignant lymphoma. The mean age of dogs with malignant tumors was 6 years.

DISEASES INVOLVING THE T CELL (CELL-MEDIATED) IMMUNE SYSTEM

Hypotrichosis with Thymic Aplasia (Nude Kittens)

An autosomal recessive disease has been described in Birman cats⁷ that appears to be the homologue of the T cell immunodeficient nude mouse and, more recently, T cell immunodeficient human nude patients. The disease is characterized by lack of hair growth and thymic development, and severe immunodeficiency. Kittens are born with no hair and fail to thrive, which results in death within a few days. Necropsy findings include lack of a thymus and severely aplastic lymph nodes with severe depletion of paracortical (T cell dependent) regions. The genetic defect in T cell deficient nude mice and human patients is a mutation in the *whm* (*winged-helix-nude*) gene that encodes a member of the forkhead/winged-helix transcription factor family with restricted expression in the thymus and skin.¹⁵

T Cell Deficiency in Growth Hormone Deficient Dogs

A growth hormone deficiency has been reported in an inbred family of Weimaraner dogs that also had an

associated T cell deficiency.²⁴ This is not surprising because there is increasing evidence that the thymus is a target organ for growth hormone.²⁵ Growth hormone is produced intrathymically by both thymic epithelial cells and thymocytes. Exogenous growth hormone enhances proliferation of and secretion of cytokines by thymic epithelial cells, and proliferation and differentiation of thymocytes. This is most likely mediated by IL-7 secreted by thymic epithelial cells.

Affected puppies appear normal at birth, but at 6–7 weeks of age they develop a wasting syndrome that is characterized by anemia, emaciation, lethargy, growth failure, and persistent infections, resulting in death. The peripheral T cells from affected dogs fail to respond to mitogenic stimulation. Serum immunoglobulin concentrations in affected dogs are within the reference interval, and the dogs are capable of producing specific antibody following immunization. The thymus is small and lacks cellularity. This syndrome is similar to that seen in several strains of immunodeficient mice. Replacement therapy with growth hormone results in marked increase in size and cellularity of the thymus and marked clinical improvement.²³

Lethal Acrodermatitis in Bull Terriers

An acquired T cell deficiency has been described in Bull terriers secondary to an abnormality in absorption and metabolism of zinc.¹⁷ The clinical signs include skin lesions (acrodermatitis, chronic pyoderma, and paronychia), diarrhea, and recurrent respiratory infections. Affected dogs also have a lighter pigmentation at birth that becomes progressively more noticeable with age. Growth retardation also is noticeable by 5–6 weeks of age when compared with their unaffected littermates. As affected puppies age they exhibit abnormal behavior characterized by diminished response to external stimuli and general activity. The median survival time for affected dogs is 7 months, with bronchopneumonia as the major cause of death. Immunological evaluation of affected dogs reveals normal immunoglobulin levels and severely depressed T cell response after mitogenic stimulation. Pathologic findings, in addition to skin pathology, include a small, frequently unidentifiable thymus, severe depletion of lymphocytes in the T cell areas of the lymph nodes and spleen, and dilatation of the cerebral ventricles. The mode of inheritance is autosomal recessive. Unlike other zinc deficiencies, affected dogs show little clinical improvement after zinc supplementation.

Viral Infections

Many viral infections are capable of producing an acquired immunodeficiency in dogs and cats, especially those that affect the lymphoid tissue. The viruses include canine distemper virus, canine parvovirus, feline leukemia virus, feline immunodeficiency virus, and feline panleukopenia virus. These viral infections cause primarily a T cell deficiency as the result of a

direct lytic effect on lymphocytes or disruption in the normal immunoregulatory cytokine network.

DEFICIENCIES OF THE COMPLEMENT SYSTEM

C3 Deficiency

C3 is a component of the complement system important in the opsonization of bacteria. A C3 deficiency, with an autosomal recessive mode of inheritance, has been reported in Brittany spaniels.⁴ Dogs that are homozygous for the trait have no detectable C3, whereas dogs that are heterozygous have C3 concentrations that are approximately 50% of normal and are clinically normal. The genetic defect is a deletion of cytosine at position 2,136 causing a frame-shift and generation of a premature stop codon resulting in a truncated protein <40% of the wild-type protein.[†]

Clinical signs are observed only in dogs that are homozygous for the C3 deficiency and are related to an increased susceptibility to bacterial infections, including septicemia, and renal disease. Older C3 deficient dogs may develop type 1 membranoglomerulonephritis and amyloidosis. The major immunologic abnormality is the absence of serum C3.

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Severe Combined Immunodeficiencies

STEVEN E. SUTER

Severe Combined Immunodeficiency in Arabian Foals

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Acronyms and Abbreviations

ADA, adenosine deaminase; DNA-PK_{CS}, DNA-dependent protein kinase; EGFP, enhanced green fluorescent protein; FPS, Fell pony syndrome; IL2RG, interleukin-2 receptor common gamma chain; NK, natural killer; PCR, polymerase chain reaction; SCID, severe combined immunodeficiency; X-SCID, X-linked severe combined immunodeficiency.

Severe combined immunodeficiency (SCID) disorders are a rare (~1/100,000 live births) heterogeneous group of at least 11 congenital genetic conditions in humans characterized by profound defects in both humoral and cell-mediated immune responses, most often leading to infantile death.⁷ SCID classification is based on four main disease mechanisms: (1) premature cell death due to accumulation of purine metabolites, seen in adenosine deaminase (ADA) deficiency; (2) defective cytokine-dependent survival signaling in T cell precursors, seen in common gamma (γ c) chain or JAK3 mutations; (3) defective V(D)J rearrangements of T cell (TCR) and B cell (BCR) receptors, seen in RAG1 or RAG2 mutations; (4) defective pre-TCR and TCR signaling, seen in CD3 δ or CD45 mutations.

Regardless of the mechanism, affected individuals are characterized by: (1) severely reduced numbers and functional capacities of T cells and, in some cases, B cells and natural killer (NK) cells; (2) failure to thrive; and (3) present with diarrhea and chronic infections after 2

months of age as protective maternal antibody titers decline. Most forms of SCID exhibit autosomal recessive inheritance with the X-linked recessive form of SCID (XSCID) being the most common (~50%). Both SCID and XSCID have been reported in the veterinary literature.

SEVERE COMBINED IMMUNODEFICIENCY IN ARABIAN FOALS

Severe combined immunodeficiency in Arabian foals, a uniformly fatal disease first reported in 1973,¹⁶ is an autosomal recessive disorder limited to foals in which both dam and sire are descendants of Arabian horses.²⁰ SCID foals appear deceptively normal at birth, but they cannot generate antigen-specific immune responses¹⁷ because they lack mature B and T cells. They have a profound lymphopenia and hypoplasia of their lymphoid organs.^{14,15,29}

Molecular Basis of Disease

SCID in Arabian foals is caused by a mutation in a gene located on chromosome 9¹ encoding the catalytic subunit of DNA-dependent protein kinase (DNA-PK_{CS}), leading to a complete lack of expression of an active version of this enzyme.²⁴ DNA-PK_{CS} is an integral enzyme involved in the V(D)J recombination events of B and T cells antigen receptor rearrangements. A lack of V(D)J recombination results in B cells lacking expression of cell surface immunoglobulin M (IgM) and T cells lacking cell surface expression of antigen-specific receptors. These defective progenitors, which are unable to complete the maturation process, are eliminated resulting in profound lymphopenia. Therefore, this disease is similar mechanistically to human RAG1 or RAG2 deficient SCID where both B and T cells are affected with sparing of NK cells.

Sequence analysis revealed the same five base pair deletion in all cases.²⁴ The deletion causes a frameshift mutation at codon 3,155 and formation of a premature stop codon at amino acid position 3,160 (of 4,127 amino acids). The resulting 967 amino acid loss eliminates the protein kinase active sites which renders the enzyme functionally inactive.

Clinical Features

Most affected foals remain free of infections and grow well for 3–8 weeks if adequate colostrum is ingested. As maternal antibodies begin to wane, respiratory infections, which typically manifest as mucopurulent nasal and ocular discharge and respiratory abnormalities, become increasingly evident. Although initially responsive to antimicrobial therapy, the respiratory infections tend to increase in severity and frequency with a declining response to therapy. Affected foals lose body condition and inevitably die between 2 and 5 months of age.

A number of opportunistic infectious agents have been isolated from SCID foals, with equine adenovirus being the most frequent and important. Infection usually starts in the upper respiratory tract or intestinal epithelial cells, with spread to other sites including bronchiolar, renal, and pancreatic duct epithelial cells. The pancreas from a SCID foal infected with adenovirus has marked reduction in exocrine tissue and an increase in fibrous connective tissue, most likely contributing to the growth reduction and loss of condition in these patients. Notable opportunistic respiratory bacteria cultured from SCID foals include *Pneumocystis carinii* and *Rhodococcus equi*. *Cryptosporidium parvum* infection can cause severe, life-threatening diarrhea and, when concomitant with

respiratory disease, can dramatically shorten the time of onset of initial infection to death. Because SCID foals have normal NK cell numbers and activity, equine herpes viruses rarely cause serious infections.

Diagnosis

Foals with selective IgM deficiency (see Chapter 56) or failure to absorb adequate maternal immunoglobulin from colostrum can have clinical signs similar to SCID foals, although profound lymphopenia (>1,000 lymphocytes/ μ L of blood) and lymphoid tissue hypoplasia is not seen. Therefore, before 1997, a definitive diagnosis of SCID required demonstration of (1) profound lymphopenia, (2) absence of IgM in serum, and (3) hypoplasia of thymus and spleen. A tentative diagnosis could be reached by analyzing blood samples, but definitive diagnosis could only be confirmed by post-mortem histologic examination of the spleen and lymphoid tissue.

The definitive molecular criteria of SCID diagnosis in Arabian foals were published in 1997.²⁴ DNA isolated from blood or buccal swabs from horses of any age is subjected to polymerase chain reaction (PCR) using primers that produce a product that spans the area of the five base pair deletion.^{24,25} DNA from a normal horse produces a 130 bp amplicon, while DNA from a SCID horse produces a 125 bp amplicon. Horses heterozygous for the disease will produce amplicons of both sizes (Fig. 57.1). Using this test the diagnosis of SCID can be reached well before the expected time of onset of infectious diseases in immunodeficient foals. Importantly, this test can be used to unequivocally determine the definitive genotype of any horse and rule in or out SCID in foals with clinical signs such as recurrent infections or failure to thrive. The diagnostic test for SCID is available from VetGen Inc. (<http://www.vetgen.com>). SCID test kits can be ordered from the Arabian Foal Association (<http://www.foal.org/TestKitsandForms.html>).

Treatment

Unfortunately, SCID is uniformly fatal, with all foals incapable of generating antigen-specific immune responses to prevent or recover from opportunistic bacterial or viral infections. Although the clinical course of the disease can be prolonged with plasma transfusions as a source of antibodies in conjunction with antimicrobial therapy, SCID foals do not survive past 5 months of age when maintained under standard equine husbandry conditions.

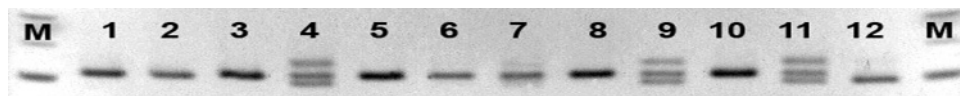


FIGURE 57.1 Genomic DNA PCR analysis for normal and SCID DNA-PK_{CS} sequences.* Lanes 1–9, clinical blood samples; lane 10, known unaffected foal; lane 11, known heterozygous foal; lane 12: known SCID affected foal; M, DNA marker. The foals in lanes 4 and 9 are heterozygous SCID carriers.

Bone marrow transplantation of a 32-day-old SCID foal using histocompatible, mixed-leukocyte-culture-nonreactive, sex-matched, full sibling marrow cells resulted in successful engraftment and reversal of the SCID phenotype.¹⁹ Peripheral blood lymphocyte counts increased within 2 weeks of transplantation and, although a full year was required for normalization of immune functions, the foal developed the ability to respond to vaccine antigens and to resist infections, indirectly implying normal B and T cell function. The horse survived for 5 years before succumbing to an intestinal disorder unrelated to SCID or bone marrow transplantation. This experiment demonstrated that hematopoietic precursors in the donor marrow with a normal copy of DNA-PK_{CS} gene have a selective advantage in a SCID recipient with a rapid expansion of functional, mature B and T cells in bone marrow, thymus, and peripheral lymphoid organs. Equine bone marrow transplantation is, unfortunately, not currently a realistic solution for the treatment of SCID mainly due to expense, technical issues relating to marrow harvesting, and the need for a histocompatible donor.

Controlling SCID in the Arabian Horse Population

SCID in Arabian horses could be completely eliminated through careful genetic screening and selective breeding based on these results. Because only SCID foals can be produced when both the sire and dam are heterozygous for the mutant DNA-PK_{CS} gene, breeding programs can be structured to ensure that heterozygous stallions are never mated to heterozygous mares. Therefore, Arabian mares and stallions should be tested and classified as homozygous, heterozygous or normal for the DNA-PK_{CS} gene. Theoretically, barring any new spontaneous mutations, Arabian SCID could be completely eliminated from the population within a few generations. Canine XSCID was eliminated from the canine breeding population using this strategy.

Although not recommended, an alternative to breeding only homozygous normal horses would be the continued use of known heterozygous horses under carefully controlled conditions based on genetic screening. For example, a heterozygous SCID stallion could breed with a homozygous normal mare with subsequent testing of all the foals to determine the genotype. The homozygous normal foals could be selected for future breeding programs while the heterozygous colts could be gelded and all heterozygous offspring be used for nonreproductive activities. For a program such as this to be successful in controlling Arabian SCID, strict adherence to these guidelines is paramount.

FELL PONY SYNDROME

Fell pony syndrome (FPS), first described in an ancient breed of ponies bred mainly in upland areas of the UK,²³ is a uniformly fatal disease also seen in Europe³ and the USA.⁸ Similar to other primary immunodeficiencies,

affected foals are usually normal at birth, but fail to thrive as maternal antibodies begin to wane. Unpublished genealogical studies suggest a possible autosomal recessive mode of inheritance, but the etiology, incidence, and pattern of inheritance of FPS have not been fully elucidated.

Clinical Presentation

At 4–8 weeks of age, affected foals begin to lose body condition and develop multiple, chronic infections that do not respond appropriately to antimicrobial therapy. Loss of body mass, diarrhea, bilateral nasal or oculonasal mucopurulent discharge, hypersalivation, coughing, and bronchopneumonia are the most frequent clinical signs. Other notable abnormalities include pyrexia, frequent chewing movements, halitosis, and a pale, friable, pseudomembranous and hyperkeratotic lingual coating. Severe normocytic normochromic anemia and lymphopenia are the most frequent blood dyscrasias. The bone marrow typically has a reduction in erythroid precursor cells. The foals deteriorate progressively and die or are euthanized, usually between 1 and 3 months of age.

Postmortem examination invariably reveals multi-systemic necrotizing and inflammatory lesions associated with opportunistic infections with a wide variety of bacteria.²² Rhinitis, sinusitis, bronchitis, pneumonia, and glossal hyperkeratosis are the most frequent respiratory abnormalities. The most frequent gastrointestinal abnormalities include hepatitis, typhlocolitis, adenovirus-induced pancreatitis, and enteritis. Most ponies also have inconspicuous lymphoid tissue including small thymuses, a lack of lymph node follicles and germinal centers, and a paucity of splenic corpuscular tissue. Peripheral gangliopathy, characterized by neuronal chromatolysis and nuclear pyknosis of dorsal root ganglia, also has been documented.²³

Immunologic Abnormalities

Fell pony syndrome foals have low IgM and IgA immunoglobulin levels,⁸ while IgG levels can be variable, most likely reflecting the presence of maternally derived IgG antibodies from colostrum. Importantly, FPS foals have significantly reduced peripheral B cell numbers (<10%) when compared to age-matched normal Fell ponies. This correlates with immunohistochemical data showing a severe reduction of CD79a+ B cells in the lymph nodes, spleen, and bone marrow from FPS foals.²³ Lymphocyte function tests and the distribution of CD4+ or CD8+ T cells are typically normal, although depressed mitogenic responses have been described.⁸ Lymphocyte cell surface expression of MHC class II is variable.

Diagnosis

At present, a definitive diagnosis of FPS relies on postmortem examination of lymph nodes, thymus, bone

marrow, and spleen. Clinical signs in a Fell pony, such as failure to thrive and recurrent infections beginning at 4–6 weeks of age can raise the level of suspicion of FPS. This, in conjunction with flow cytometric examination to determine if B cell numbers in peripheral blood are less than 10% of that in normal foals, may be used ante mortem to support the diagnosis of FPS.

Treatment

As with most primary immunodeficiencies in animals, there is no treatment for FPS. This disease probably is the result of a severe defect in B cell development at the level of the bone marrow, which contributes to failure of B cell expansion in the periphery. Implementation of appropriate breeding programs to reduce the incidence of FPS awaits elucidation of the underlying genetic lesion(s), proof of genetic inheritance, and the development of a carrier test, similar to SCID in Arabian foals.

X-LINKED SEVERE COMBINED IMMUNODEFICIENCY (XSCID)

X-linked recessive SCID (XSCID), the most frequent SCID in humans,⁴ also has been reported in dogs.^{6,9,13,21,27} Both species share common immunologic abnormalities including: peripheral T cell lymphopenia with variable numbers of phenotypically normal B cells that do not undergo isotype class-switching to IgG in vivo; peripheral T cells that do not proliferate in response to mitogens; and reduced production of NK cells.¹⁸ This is in contrast to SCID in Arabian foals where patients have severely reduced peripheral B and T cell counts and normal NK activity. Similar to SCID Arabian foals, human and canine XSCID patients have reduced capacity to prevent or combat opportunistic infections resulting in infantile death.

Canine XSCID

Incidence of Canine XSCID

Canine XSCID was first reported in a litter of Basset Hounds in 1989.¹³ Breeding studies confirmed the disease was X-linked. Additional cases of XSCID in the general Basset Hound population have not been reported. Canine XSCID was again recognized in 1997 in closely related Cardigan Welsh Corgi dogs.²¹ Pedigree analysis of this family was consistent with X-linked inheritance, and the definitive diagnosis of XSCID was confirmed with DNA analysis. Subsequent pedigree DNA analysis identified the gene mutation in an affected male dog, related obligate carrier females, and grandmother of the affected dog. Subsequent genetic testing identified carrier females so they could be removed from the breeding population, which eliminated spread of the disease.

The genetic mutations responsible for XSCID in Basset hounds and Cardigan Welsh Corgis are probably

due to random chance. Since genetic testing followed by controlled breeding has eliminated the disease from the Corgi and Basset populations, XSCID is no more likely to occur in these two breeds than in any other breed.

Molecular Basis of Canine XSCID

Similar to XSCID affected boys, canine XSCID is caused by mutations in the gene encoding the third or gamma chain of the interleukin-2 (IL-2) receptor.^{12,26} The IL-2 receptor gamma chain also is a component of the cytokine receptors for IL-4, IL-7, IL-9, and IL-15.¹⁵ The normal γ protein, which is 373 amino acids in length and 84% and 71% identical to the human and mouse protein sequences, respectively,¹² is composed of several evolutionarily conserved potential functional domains. Unlike human XSCID where hundreds of different γ mutations have been identified only two mutations have been identified in dogs. Basset Hounds have a four base pair deletion in the signal peptide region of the γ gene that causes a shift in the translation reading frame and premature truncation of the γ protein during its synthesis. The mutant, nonfunctional protein is only 21 amino acids in length.¹² Cardigan Welsh Corgi dogs have a single nucleotide insertion following nucleotide 582 that also causes a shift in the translation reading frame and premature truncation of the γ protein at amino acid 196. This effectively eliminates the transmembrane and the Src homology domains, resulting in a nonfunctional protein.

Clinical Presentation

Similar to XSCID boys, canine XSCID is characterized by a failure to thrive and an increased susceptibility to bacterial and viral infections at a young age.¹³ Puppies appear normal at birth and are presumably protected from overwhelming infection by maternal antibodies for several weeks thereafter. As maternal antibodies wane, XSCID pups begin to lag behind their normal littermates in size and body mass and infections begin to develop. These infections include a wide range of opportunistic organisms that are rarely responsible for disease in normal animals. Bacterial pyoderma, otitis, and cystitis are common, as well as diarrhea secondary to gastrointestinal infections with various parasites (*Giardia*, *Coccidia*, *Campylobacter*, *Cryptosporidium*). Affected pups lack palpable peripheral lymph nodes and no thymic shadow is visible on thoracic radiographs. Antimicrobial therapy is generally ineffective in combating recurrent infections and puppies die or are euthanized by 5–6 months of age.

The thymuses of XSCID pups are frequently less than 10% of the mass of age-matched unaffected littermates and are usually obscured by adipose tissue. Histologically, thymuses from XSCID pups can range from simple dysplasia with no Hassall's corpuscles to relatively normal looking with many Hassall's corpuscles, although total thymocyte numbers are always reduced.

Immunologic Abnormalities

The most significant immunologic problem in XSCID is the defect in T cell function and development. XSCID puppies are variably lymphopenic, with normal or increased percentages of B cells, and usually decreased percentages of peripheral T cells. XSCID dogs can develop variable numbers of phenotypically mature peripheral T cells; their ability to proliferate in response to mitogenic stimulation or IL-2 binding is negligible due to a dysfunctional IL-2 receptor.

Treatment of XSCID

In humans, bone marrow transplantation is the only treatment for XSCID, with success ranging from 50% to nearly 100%. Gene therapy using retroviral vectors harboring a normal copy of the γ c cDNA has cured a number of affected boys although two patients later developed leukemia, probably the result of insertional mutagenesis.¹⁰

XSCID puppies have received bone marrow transplants using unfractionated whole marrow collected from both normal and carrier littermates.⁵ The transplanted dogs, none of which received pre-transplant cytoablative therapy, developed completely engrafted donor T cells, partially engrafted donor B cells, and were maintained in a conventional environment. XSCID puppies have also been transplanted with highly purified populations of CD34+ hematopoietic progenitor cells harvested from DLA-matched (dog leukocyte antigen) littermates.¹¹ All six dogs in this study went on to develop completely engrafted donor T cells from 1 to 3 months after transplantation, variable engrafted donor B cells, and were maintained in a conventional environment for over 6 months.

Retroviral gene therapy has been used to treat canine XSCID. In an initial study, a murine retroviral expression vector harboring enhanced green fluorescent protein (EGFP) and γ c cDNAs was used for the ex vivo transduction of normal DLA-matched CD34+ cells followed by transplantation into XSCID affected littermates.²⁸ Three of the five transplanted dogs went on to develop prolonged immune reconstitution with up to 28% of peripheral blood T cells expressing EGFP. A more recent study used in vivo retroviral gene therapy whereby XSCID dogs were directly injected with a retroviral vector encoding a normal copy of the γ c cDNA. Three of the four injected dogs attained normal numbers of gene-corrected peripheral T cells by 8 weeks after treatment.

SEVERE COMBINED IMMUNODEFICIENCY IN JACK RUSSELL TERRIERS

Severe combined immunodeficiency also has been reported in a single family of Jack Russell terriers. Twelve of 32 siblings from this single pair breeding succumbed to opportunistic infections within 8–14 weeks of age. Additional breedings permitted the elu-

cidation of the underlying genetic defect responsible for the immunodeficiency.

Molecular Basis of Disease

Jack Russell terriers SCID that is inherited as an autosomal recessive trait, is a non-B/non-T SCID probably caused by a genetic DNA-PK_{CS} deficiency, similar to Arabian foal SCID.^{2,17} A single nucleotide polymorphic transition (A/G) at position 95 of intron 5 results in a stop codon at nucleotide 10,828 and a premature termination at amino acid 517. This functionally null allele is responsible for lack of DNA-PK_{CS} activity and subsequent defective V(D)J recombination. The carrier frequency of the Jack Russell terriers SCID allele is less than 1.1%.

Clinical Features

The clinical features of SCID in Jack Russell terriers mirror those seen in most other SCID affected animals. Pups typically appear normal until maternal antibodies begin to wane, with most succumbing to a wide variety of opportunistic infections at 8–14 weeks of age. Extreme lymphopenia and agammaglobulinemia are seen, while thymic dysplasia, and peripheral lymphoid aplasia are the most frequent findings at necropsy.

Diagnosis

To this author's knowledge, standard testing of Jack Russell terrier SCID is not currently available.

Treatment

There are no published reports of Jack Russell terrier SCID treatment, although bone marrow transplantation using DLA-matched siblings is, at least theoretically, a possibility.

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Benign Lymphadenopathies

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Acronyms and Abbreviations

CBC, complete blood count; CLL, chronic lymphocytic leukaemia; FeLV, feline leukaemia virus; FIP, feline infectious peritonitis; FIV, feline immunodeficiency virus; IL-1, interleukin-1; RMSF, Rocky Mountain spotted fever; TNF, tumor necrosis factor.

Lymph nodes constitute a major component of the mononuclear phagocytic and immunologic systems. Because of their dynamic state, they constantly reshape and change in size in response to various stimuli. Most tissue changes within these structures result in lymph node enlargement. This enlargement frequently represents the only abnormality on physical examination in an otherwise healthy patient, and alerts the clinician to an ongoing disease process.

LYMPH NODE ANATOMY

The microscopic architecture of the lymph node is reviewed in Chapter 52 and elsewhere.^{9,29} From a clinical standpoint, it is important to become familiar with the characteristics of palpable lymph nodes in normal dogs and cats, so that subtle changes can be detected. Palpable lymph nodes in dogs and cats include the mandibular, prescapular (or superficial cervical), axillary, superficial inguinal, and popliteal. Other lymph nodes become palpable only when enlarged; these include the facial, retropharyngeal, and iliac (or sublumbar) nodes.⁹

LYMPHADENOPATHY

Definitions

In the context of this chapter, the term lymphadenopathy refers to lymph node enlargement, although lymph node atrophy, for example, should also be considered a lymphadenopathy. Lymph node involution has been described in cases of chronic stress, anticancer chemotherapy, radiation, but also in the course of some viral infections, like parvoviral infections in young puppies or in the end stage of feline immunodeficiency virus (FIV) infection in cats.¹⁴

Solitary or isolated lymphadenopathy refers to enlargement of a single lymph node. Regional lymphadenopathy describes the enlargement of more than one lymph node draining a specific anatomic area; these nodes are usually interconnected. Generalized lymphadenopathy refers to multicentric lymph node enlargement affecting more than one anatomic area. Lymphadenopathies can also be classified as superficial or deep, according to their anatomical location.

Pathogenesis

Lymph nodes are in a dynamic state that results in constant reshaping. Lymph node enlargement usually occurs as a consequence of either enhanced proliferation of normal cells within the node or infiltration with normal or abnormal cells. When normal cells proliferate within a node, the term reactive lymphadenopathy is used. Proliferation of normal lymphoid or mononuclear phagocytic cells occurs in response to various stimuli, mainly infectious and immunologic, although occasionally the clinician will encounter a dog or cat with reactive lymphadenopathy in which an etiologic agent cannot be identified (i.e. idiopathic reactive lymphadenopathy). Basically the lymph node can react in three ways: (1) by hyperplasia of the follicles (B cell response); (2) by hyperplasia of the paracortical area (T cell response); and (3) by hyperplasia of the mononuclear histiocytic cells in the medullary sinuses. In the majority of cases the reaction is nonspecific leading to nonspecific reactive hyperplasia.

When polymorphonuclear leukocytes or inflammatory macrophages predominate in the lymph node infiltrate, the term lymphadenitis is used; this process frequently occurs secondary to infectious agents. If neutrophils predominate, the lymphadenitis is considered to be suppurative; if macrophages are the predominant cell, the inflammation is granulomatous; if macrophages and neutrophils occur, the term pyogranulomatous lymphadenitis is preferred.⁹ Infectious diseases associated with lymphadenopathy in dogs and cats and their typical cellular infiltrate on lymph node cytology are listed in Table 58.1.

Infiltrative lymphadenopathies result from displacement of normal lymph node tissue by neoplastic or inflammatory cells. Inflammatory lymphadenopathies are classified by the predominant cell type present. Neoplasms involving the lymph nodes can be either primary hematopoietic malignancies or secondary (metastatic) neoplasms. Neoplastic causes of lymphadenopathy are discussed in Section V. Table 58.1 provides a classification scheme for lymphadenopathy in dogs and cats.

TABLE 58.1 Classification of Lymphadenopathy in Dogs and Cats

Type	Species ^a	Type	Species ^a
Proliferative Lymphadenopathies			
<i>Infectious</i>			
Bacterial (neutrophilic/suppurative) ^b		Babesiosis	D
Streptococci	C, D	Toxoplasmosis	C, D
<i>Corynebacterium</i>	C	Viral (cellular infiltrate varies with agent) ^b	
<i>Brucella</i>	D	Infectious canine hepatitis	D
Mycobacteria	C, D	Canine herpesvirus	D
<i>Actinomyces</i> spp.	C, D	Canine viral enteritides	D
<i>Nocardia</i> spp.	C, D	Feline retroviruses	C
Localized bacterial infection	C, D	Feline infectious peritonitis	C
<i>Yersinia pestis</i>	C	<i>Noninfectious</i>	
<i>Francisella tularensis</i>	C, D	Postvaccinal	C, D
<i>Bartonella</i> spp.	C, D	Immune-mediated disorders	
Rickettsial (plasmacytic) ^b		Systemic lupus erythematosus	D
Ehrlichiosis	D, C	Rheumatoid arthritis	D
Anaplasmosis	D, C	Immune-mediated polyarthritides	C, D
Rocky Mountain spotted fever	D	Localized inflammation	C, D
Salmon poisoning	D	Aluminosilicate	D
Fungal (granulomatous/pyogranulomatous) ^b		Idiopathic	C, D
Histoplasmosis	C, D	Infiltrative Lymphadenopathies	
Blastomycosis	C, D	<i>Neoplastic</i>	
Cryptococcosis	C, D	Primary Hematopoietic Neoplasms	
Coccidioidomycosis	C, D	Lymphomas	C, D
Aspergillosis	C, D	Histiocytic sarcomas	D
Sporotrichosis	C, D	Leukemias	C, D
Phaeohyphomycosis	C, D	Multiple myeloma	C, D
Phycomycosis	C, D	Systemic mast cell disease	C, D
Algal (granulomatous/pyogranulomatous) ^b		Metastatic Neoplasms	
Protothecosis	C, D	Carcinomas	C, D
Parasitic (cellular infiltrate varies with agent) ^b		Sarcomas	C, D
Demodicosis	C, D	Mast cell tumors	C, D
Trypanosomiasis	D	Malignant melanomas	D
Leishmaniasis	D	<i>Non-Neoplastic</i>	
Hepatozoonosis	D	Mast cell infiltration (non-neoplastic)	C, D
		Eosinophilic granuloma complex	C, D?
		Lysosomal storage diseases	D, C?

^aC, cats; D, dogs.

^bTypical cellular infiltrate on cytology.

EVALUATION OF THE PATIENT WITH LYMPHADENOPATHY

Thorough evaluation of the patient with lymphadenopathy is essential for establishing an accurate diagnosis. Several reviews of this subject are available,^{9,30} but brief summary of proper patient evaluation is included below.

Historical and Physical Examination Findings

Several clues of diagnostic value can be obtained from the history. A detailed travel history should be obtained in dogs presented for evaluation of generalized lymphadenopathy, because certain diseases have a definite geographical distribution (e.g. ehrlichiosis in the southwestern or southeastern United States). Other diseases associated with lymphadenopathy have a seasonal distribution (e.g. Rocky Mountain spotted fever [RMSF] in spring and summer). A vaccination history should also be obtained, because generalized reactive lymphadenopathy shortly after vaccination is relatively frequent. In addition, lymphadenopathy after administration of certain drugs also has been reported.²⁵

The presence or absence of systemic clinical signs in dogs or cats with generalized lymphadenopathy is helpful in guiding the clinician to a specific diagnosis, because severe systemic signs are more frequently associated with certain diseases, such as systemic mycoses, salmon poisoning, RMSF, leishmaniasis, and acute leukemias. In contrast, systemic signs can be absent or are mild in dogs and cats with lymphoma or chronic leukemia.

The distribution of lymphadenopathy is also of diagnostic relevance. When evaluating a patient with solitary or regional lymphadenopathy, the clinician should focus attention on the area drained by the lymph node or nodes, because the primary lesion probably will be found there. In contrast, most dogs and cats with generalized lymphadenopathy have systemic fungal or rickettsial infections, idiopathic lymph node hyperplasia, or hematopoietic neoplasia. Cats and dogs differ in their distribution pattern of peripheral malignant lymphoma. Where dogs usually have a generalized lymphadenopathy, cats usually have solitary or regional lymph node enlargement.

The palpable characteristics of the nodes may give additional information. In most instances of lymphadenopathy, lymph nodes are firmer than usual, are irregular, painless, do not feel warm, and do not adhere to the surrounding tissues. The main exception to this rule occurs in patients with lymphadenitis in which the nodes feels softer, tender (i.e. painful lymphadenopathy), warmer than normal, and adhere to surrounding structures (i.e. fixed lymphadenopathy).

From the clinical standpoint, the size of the affected lymph nodes is important. In our experience, marked lymphadenopathy (i.e. lymph node size 5–10 times normal) occurs almost exclusively with lymphadenitis (e.g. lymph node abscessation and tuberculosis) and

with lymphomas. Rarely, metastatic lymph nodes enlarge to this degree. Dogs that have salmon poisoning also may present with marked generalized lymphadenopathy preceded by (or in conjunction with) bloody diarrhea.¹⁶ Cats that have idiopathic reactive lymphadenopathy usually have significant generalized lymph node enlargement.^{21,23,24} Mild lymph node enlargement (i.e. 2–4 times normal size) occurs most frequently in association with various reactive lymphadenopathies and lymphadenitides (e.g. ehrlichiosis, RMSF, systemic mycoses) and in leukemias.^{5,18–20}

As noted, a thorough examination of the area or areas draining the enlarged lymph node or nodes should be performed, paying particular attention to the skin, subcutis, and musculoskeletal structures. In patients that have generalized lymphadenopathy, it is important to evaluate other hemolymphatic organs, including the spleen, liver, and bone marrow.

Laboratory Evaluation

Obtaining a complete blood count (CBC) and a serum chemistry profile are of importance, particularly during evaluation of patients that have generalized lymphadenopathy. Changes in the CBC may suggest a systemic inflammatory process (e.g. leukocytosis caused by neutrophilia, left shift, and monocytosis in a dog with histoplasmosis or blastomycosis) or a diagnosis of hematopoietic neoplasia (e.g. presence of circulating blasts or marked lymphocytosis suggestive of chronic lymphocytic leukemia). Infrequently the etiologic agent can be identified by examining a blood or buffy coat smear (e.g. histoplasmosis, trypanosomiasis, babesiosis, ehrlichiosis).

Anemia in patients with lymphadenopathy can be caused by several mechanisms. Anemia of inflammatory disease can be seen in inflammatory, infectious, or neoplastic disorders; hemolytic anemia is usually present in patients with hemoparasitic lymphadenopathies or histiocytic/lymphoid malignancies; severe nonregenerative anemia can be observed in dogs that have chronic ehrlichiosis or immune-mediated disorders, in cats that have retroviral infections, and in dogs and cats that have primary bone marrow neoplasms (i.e. leukemias). Intra-abdominal lymphadenopathy in a dog that has relative iron deficiency anemia indicates the presence of a gastrointestinal neoplasm.^{3,10} The reticulocyte hemoglobin content may help in these cases as a marker of iron deficiency.

Thrombocytopenia is a frequent finding in patients that have ehrlichiosis, RMSF, sepsis, lymphoma, leukemias, multiple myeloma, systemic mastocytosis, malignant histiocytosis, retroviral infections, and some immune-mediated disorders.^{10–13,22,27,34,37} Pancytopenia is frequent in dogs that have chronic ehrlichiosis, histiocytoses, and systemic immune-mediated disorders; in dogs and cats that have leukemia; and in cats that have retroviral disorders.^{5,17–20,37}

Two major serum chemistry abnormalities are of diagnostic value in dogs and cats that have lymphadenopathy are hypercalcemia and hyperglobulinemia.

Hypercalcemia can result from a paraneoplastic syndrome that occurs in approximately 10–20% of dogs that have lymphoma³⁴ or multiple myeloma;²² it also has been documented in dogs with blastomycosis.¹³ Hypercalcemia is rare in cats; neoplasms associated with hypercalcemia in this species include lymphoma, squamous cell carcinoma, fibrosarcoma, myeloma, and parathyroid tumors.^{8,32} Monoclonal hyperglobulinemia can occur in dogs and cats that have multiple myeloma,^{8,22} in dogs that have chronic lymphocytic leukemia (CLL),²⁰ and infrequently in dogs that have lymphoma,³⁴ ehrlichiosis,⁴ or leishmaniasis.¹⁵ Polyclonal hyperglobulinemia, however, is more frequently associated with infectious diseases like leishmaniasis, ehrlichiosis and systemic mycoses, and in cats with feline infectious peritonitis (FIP).^{1,4,8,18}

Serologic testing (e.g. FIV, *Leishmania*, *Toxoplasma*, *Ehrlichia*), direct antigen tests (e.g. FeLV), or PCR techniques (e.g. FIP, *Ehrlichia*) are indicated when infectious causes for the lymphadenopathy are suspected.

Imaging

Radiographic abnormalities in dogs that have lymphadenopathy vary with the primary disorder. Generally, plain radiographs are beneficial in patients that have deep regional lymphadenopathy involving the thoracic and the abdominal cavities. Ultrasonography is a noninvasive procedure that provides great benefit in evaluating intra-abdominal lymphadenopathy. Especially in combination with color Doppler characteristics, ultrasonography may be useful in distinguishing neoplastic from inflammatory lymphadenopathy.^{26,31} In addition, lymph nodes can be accurately imaged and measured, so that therapeutic progress can be monitored. Moreover, ultrasound-guided aspirates or biopsies can be performed in patients with minimal complications.²

Bone Marrow Findings

Evaluation of bone marrow aspirates or core biopsies can be beneficial in patients that have generalized lymphadenopathy caused by hematopoietic neoplasia or systemic infectious diseases (e.g. histoplasmosis, ehrlichiosis, leishmaniasis, see Chapter 19).

Lymph Node Aspirate

Cytologic evaluation of lymph node aspirates provides the clinician with a wealth of information and is frequently the definitive diagnostic procedure in patients that have lymphadenopathy.³⁵ Superficial lymph nodes can be aspirated with minimal difficulty; the successful aspiration of intrathoracic or intra-abdominal lymph nodes requires some expertise. For a fine needle aspirate of a superficial node to be obtained, the area does not need to be surgically prepared. Aspiration of intrathoracic and intra-abdominal structures requires surgical preparation of the area and adequate restraint

of the patient; in addition, ultrasonographic guidance can be beneficial in obtaining the desired specimen. The technique for fine needle aspiration of lymph nodes is reviewed elsewhere.⁹

Several reports of cytologic evaluation of lymphoid tissues have appeared in the veterinary literature.^{11,36} Briefly, normal lymph nodes are composed primarily of small lymphocytes (80–90% of all cells); a low number of macrophages, medium or large lymphocytes, plasma cells, and mast cells can also be found. Reactive lymph nodes are characterized by variable numbers of lymphoid cells in different stages of development (i.e. small, medium, and large lymphocytes; immunoblasts; and plasma cells). As discussed above, the cytologic features of lymphadenitis vary with the etiologic agent and the type of reaction elicited (i.e. neutrophils in suppurative inflammation; macrophages in granulomatous reactions; an admixture of both in pyogranulomatous reactions). Causal agents can frequently be identified in cytologic specimens obtained from affected nodes. Metastatic neoplasms have different cytologic features depending on the degree of involvement and the cell type. Primary lymphoid neoplasms (lymphomas) are characterized by a more monomorphic population of lymphoid cells, which are usually immature (centroblasts).³⁶

Lymph Node Biopsy

When cytologic examination of an enlarged lymph node fails to provide a definitive diagnosis, excision of the affected node for histopathologic examination is indicated. In this regard, it is preferable to excise the whole node. Core or needle biopsies are difficult to interpret because nodal architecture is not preserved. Care should be exerted in handling the node during surgical manipulation, because trauma may induce artifacts and preclude interpretation of the specimen. The popliteal lymph nodes are easily accessible, and are the ones usually excised in dogs and cats that have generalized lymphadenopathy.

Once a node is excised, it should be halved lengthwise; impression smears should be made for cytology; and the node should be fixed in neutral buffered 10% formalin, at a rate of one part of tissue to nine parts of fixative. The specimen is then ready to be referred to a laboratory for paraffin embedding and sectioning. Unfixed tissue also can be submitted in a sterile container for bacterial or fungal culture.

SELECTED DISORDERS ASSOCIATED WITH BENIGN LYMPHADENOPATHY IN DOGS AND CATS

In-depth discussion of all causes of lymphadenopathy is beyond the scope of this chapter, but Table 58.1 outlines the most frequent causes. The reader is referred to specific references regarding each condition or infectious agent. A discussion of a few conditions causing lymphadenopathy follows.

Bacterial Lymphadenitis

Two syndromes presumptively associated with pyogenic bacterial infections and lymphadenitis, contagious streptococcal lymphadenitis of cats and puppy strangles, have been described in small animals.⁸ Also, localized streptococcal and staphylococcal infections may result in solitary or regional lymphadenopathy in these species.

Contagious streptococcal lymphadenitis was described in several kittens in a cat colony.^{8,33} The kittens developed diarrhea and fever at 4 weeks of age and cervical lymphadenopathy 2 weeks later. The lymph nodes in these cats were significantly enlarged and developed draining purulent lesions. An unrelated adult cat, housed with these kittens, also developed similar signs and lesions, and the disease could be transmitted orally by administration of lymph node material from the affected cats. All the cats tested negative for circulating feline leukemia virus (FeLV) p27 antigen. Lancefield group G beta-hemolytic streptococci were isolated from abscessed lymph nodes and from heart blood in one kitten that died of septicemia. Most cats responded to treatment with penicillin.

Puppy strangles (i.e. juvenile cellulitis) is a disorder associated with cutaneous cellulitis of the head and neck, and mandibular and cervical lymphadenopathy in 4- to 12-week-old dogs.³⁸ Pups usually present with fever and facial skin lesions including edema, pustules, and crusts periorally, periocularly, on the chin or muzzle, or in the ears, along with lymphadenopathy. Sometimes the lymphadenopathy is not associated with skin lesions. More than one pup in the litter is frequently affected. Fine needle aspiration of affected lymph nodes reveals purulent lymphadenitis, and staphylococcal or streptococcal organisms may be cultured. However, due to lack of response to antibiotic therapy in a high proportion of cases, the absence of bacteria in most cultures obtained from affected areas, and the subsequent response to immunosuppressive doses of corticosteroids, a hypersensitivity reaction to staphylococcal antigens is suspected.

Idiopathic Lymphadenopathies

A syndrome, called idiopathic lymphadenopathy has been described in the cat in several reports.^{21,23,24} In one report, 14 cats, that ranged in age from 5 months to 2 years, had generalized lymphadenopathy.²⁴ The term distinctive peripheral lymph node hyperplasia was chosen by the authors for this condition. Eight cats were clinically normal on initial physical examination, except for the presence of lymphadenopathy; clinical signs in the other six cats included fever (five cats), lethargy (three cats), anorexia (three cats), pallor, hematuria, eczema, vomiting, and mastitis (one cat each). Of the 14 cats for which CBCs were available, nine had anemia; one cat had neutrophilia and lymphocytosis; and three cats had neutropenia. Six of nine cats evaluated for FeLV antigens in peripheral blood were positive.

Therapy in affected cats included antibiotics, corticosteroids, and fluids. In six cats, the lymphadenopathy resolved within 2 weeks to 4 months; one of these cats subsequently developed intrathoracic lymphoma and died; one additional cat had recurrence of the lymphadenopathy in 5 years and was euthanized; three cats had persistent lymphadenopathy and also were euthanized within a month of the initial diagnosis; four cats were lost to follow-up.⁷ Histologic changes in affected nodes included distortion of the architecture; proliferation of histiocytes, lymphocytes, plasma cells, and immunoblasts in paracortical regions; and numerous prominent postcapillary venules. These changes were similar to the ones observed in seven cats with experimental FeLV infection.⁷ Based on these changes and the fact that six of the nine cats evaluated were FeLV-positive, the authors postulated that this syndrome is secondary to retroviral infection.

In another report, six young cats were described with marked generalized lymphadenopathy resembling lymphoma.⁸ The cats ranged from 1 to 4 years in age and three of them were Main Coon cats. Most peripheral nodes were 2–3 cm in diameter and firm. Four cats had leukocytosis and one had leukopenia. One of the cats was anemic, and atypical lymphocytes were visualized in a blood smear. Five cats, evaluated for FeLV viremia, were negative. Serum protein electrophoresis in five cats revealed the presence of polyclonal gammopathies; one cat was hypercalcemic. Histological features were suggestive of lymphoma and included loss of normal lymph node architecture, presence of a uniform population of lymphoid cells in the paracortical areas, capsular and perinodal infiltration, and presence of large follicular structures without germinal centers. However, other features were not compatible with malignancy, including abundant vascularity; lymphoid follicles with active germinal centers; presence of a mixed population of lymphoid cells, plasma cells, histiocytes, and granulocytes in the sinuses. One of the cats was euthanized on presentation; the remaining five cats were not treated. Resolution of the lymphadenopathy was seen in all cats within 5–120 days and all cats were alive 12–84 months after initial diagnosis.

The clinical and pathologic features in cats that have idiopathic reactive lymphadenopathy are similar to those observed in cats naturally or experimentally infected with feline immunodeficiency virus (FIV).^{5,6} It is improbable that all cats that have idiopathic reactive lymphadenopathy are infected with FIV; more likely, FIV represents one of the agents that can induce these changes in lymph nodes of domestic cats. Indeed, in one report few differences were found between FIV-infected cats and seronegative cats with regard to lymph node pathology.²⁸

A clinical syndrome characterized by solitary cervical or inguinal lymphadenopathy in cats ranging in ages from 3 to 14 years, and referred to as plexiform vascularization of lymph nodes has been described.²¹ The lesions were unilateral in seven of nine cats studied. Most cats were asymptomatic and the FeLV status was

not reported. Surgical removal of the affected nodes resulted in uneventful recovery in most cats; however, postoperative edema occurred in two cats. Histologically, the nodes showed replacement of the interfollicular pulp by a plexiform proliferation of small capillary-sized vascular channels and lymphoid atrophy. The pathogenesis of this syndrome has not been elucidated.

Mineral-Associated Lymphadenopathy

A primary lymph node disease termed mineral-associated lymphadenopathy was described in the 1980s and has been reported since exclusively in dogs in the United Kingdom.¹² Affected dogs have nonspecific clinical signs (e.g. malaise, pyrexia, inappetence) in combination with generalized lymphadenopathy. Histologic examination of lymph nodes reveals a granulomatous lymphadenitis with accumulation of large quantities of crystalline-appearing, mineral material in the node. Electron microprobe analysis of this mineral reveals it consists of a complex mixture of elements including aluminum, silicon, titanium, and nickel, suggesting an environmental origin. In affected lymph nodes, inflammatory cytokines (IL-1, TNF- α) are produced causing the clinical symptoms. In several cases concurrent diseases, like malignant lymphoma and immune-mediated diseases, are present. Apart from treating the concurrent disease, mineral-associated lymphadenopathy frequently responds favourably to anti-inflammatory treatment.

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SECTION V

Hematologic Neoplasia

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Cell Cycle Control in Hematopoietic Cells

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The Cell Cycle Engine

Cyclins

Cyclin-Dependent Kinases

Cyclin-Dependent Kinase Inhibitors

Regulation of Cyclin-Dependent Kinase Activity

Maintenance of G₀

Negative Regulation of Cell Cycle Entry

The G₀ to G₁ Transition and G₁ Phase

Progression

The G₁ Restriction Point

Regulation of the G₁ to S Transition by Rb

S Phase Progression

Checkpoint Transit

Regulation of the Origin or Replication

G₂ Phase Progression

DNA Damage Checkpoint

Preparation for Mitosis

M Phase Progression

Prophase

Metaphase

Anaphase

The Cell Cycle and Cancer

Acronyms and Abbreviations

APC, anaphase promoting complex; CAK, CDK-activating kinase; CDK, cyclin-dependent kinase; CDKI, CDK inhibitor; DNA, deoxyribonucleic acid; G(0), gap (0) phase; HDAC, histone deacetylase; IL, interleukin; MPF, maturation promoting factor; ORC, origin of replication complex; TGF- β , transforming growth factor- β .

In the late 1980s there was a confluence of several fields of research. Scientists studying cell division in budding yeast (*Saccharomyces cerevisiae*), in fission yeast (*Schizosaccharomyces pombe*), and in frog eggs (*Xenopus laevis*) discovered a group of proteins that were responsible for cell cycle oscillation.^{4,7,15} Because the proteins were expressed cyclically, they were named cyclins. In short order, a “cell cycle engine” that functions in all eukaryotic organisms was defined. This mechanism consists of cyclin-regulated cell cycle kinases that provide the crucial checkpoints and feedback controls that regulate cell division.

THE CELL CYCLE ENGINE

To simplify its study, the cell cycle has been subdivided into various steps (Fig. 59.1). These steps include Gap (G₀), or the phase at which cells are in a resting or non-proliferative phase; G₁, which defines entry into the cell cycle and is characterized by increased cellular metabolism and growth; Synthesis (S), in which the whole genetic complement of the cell is replicated; G₂, where the cell finishes the growth processes necessary for divi-

sion; and finally mitosis (M), where division of one cell into two daughter cells finally takes place.⁸ In normal cells, transitions among these states are defined functionally and are exquisitely fine-tuned. The balanced action of proliferative and anti-proliferative signals ultimately determines whether cells enter the cell cycle, exit the cell cycle, or undergo programmed cell death (i.e. apoptosis).⁶

The cell cycle engine defines a regulatory network whose key components include proteins from two unrelated gene families, the cyclins and the cyclin-dependent kinases (CDKs). These proteins control phase transition, and are regulated transcriptionally and post-transcriptionally. In this chapter, we will use T cells to illustrate the transition steps that regulate cell cycle entry, progression, and exit.

Cyclins

Cyclins are highly conserved in all eukaryotic species studied. More than 12 mammalian cyclin homologue genes have been characterized, but here we will focus only on those genes that control cell cycle transitions. Cyclins function as regulatory subunits for the CDKs,

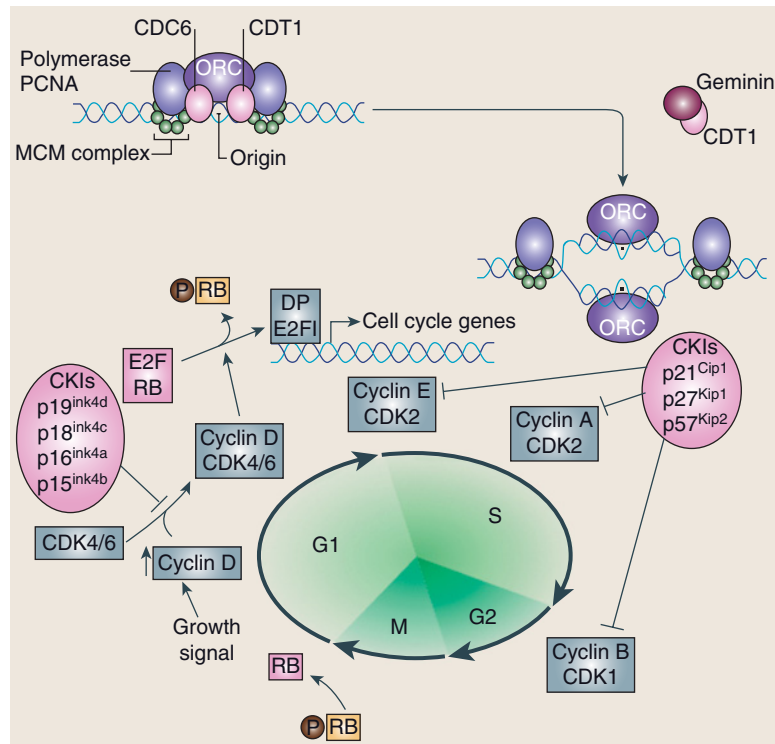


FIGURE 59.1 A schematic representation of the cell cycle. Cells enter the cell cycle in the G1 phase. G0 is a specialized form of G1 (highly differentiated cells, which are unlikely to divide unless provoked, remain in G0). During late G1 the cell commits to divide, typically marked by phosphorylation of Rb, and activation of E2F, which in association with DP1 binds to the promoters of various cell cycle genes. The process of replication of the entire genome is completed during the S phase, resulting in doubling of DNA content of the cell. The mechanical components that will organize chromosomes and physically divide the cell are assembled during the G2 phase, and a complex cytological event that accurately divides the duplicated sets of chromosomes and coordinates the events of fission to produce two cells from one is executed during the M phase. Many events take place during M phase, including dephosphorylation of RB. Upon completion of M phase, both daughter cells re-enter G1 and a new cell cycle is set to begin. Proteins that drive or inhibit the cell cycle are shown in gray or pink boxes, respectively. Cyclins are activator proteins that are up- or down-regulated depending on the phase of the cell cycle. CDKs are serine/threonine kinases that require the binding of a cyclin (or related protein) for full activity. Their range of substrates is not fully defined, but interfering with their activity arrests or slows the cycle. CDKIs are small peptides that block cyclin/CDK activity either by forming an inactive complex or by acting as a competitive CDK ligand. DNA polymerases, proliferating cell nuclear antigen (PCNA), and mini-chromosome maintenance (MCM) proteins initiate replication only once per cycle. The checkpoint proteins are members of a network of proteins that monitor DNA integrity and arrest the cell cycle until DNA damage can be repaired. (Reproduced from Herrup K, Yang Y, Cell cycle regulation in the postmitotic neuron: oxymoron or new biology? *Nat Rev Neurosci* 2007;8:368–378, with permission. ©Macmillan.)

because monomeric CDKs have essentially no catalytic function.¹⁷ However, the association of a CDK and a cyclin into a complex is only one regulatory step in the activation of these protein kinases. The cyclins are divided into groups according to the phase of the cell cycle in which they are expressed. Examples include the mitotic cyclins (B-type cyclins), S phase cyclins (cyclins A and E), and G1 cyclins (cyclins D1-3, cyclin E). Cyclin H is part of an enzyme complex called CDK-activating kinase (CAK). The nucleotide and amino acid sequence homology among cyclin proteins is limited (<30% at the amino acid level), and is restricted to a highly conserved region called the cyclin box. This cyclin box is required for association with a CDK, along with an independent, protein-specific sequence that mediates binding to small molecules called cyclin-dependent kinase inhibitors (CDKIs) that facilitate assembly of the cyclin-CDK complex.¹⁰

Cyclin-dependent Kinases

In higher eukaryotes, the CDKs comprise a family of more than 12 proline-directed, serine and threonine protein kinases that share a PSTAIRE motif. Those members of the CDK family that control cell cycle transitions are subdivided into proteins closely related to CDK1 (the mammalian Cdc2 homolog) that include CDK2, CDK3, CDK 4, CDK5, and CDK6.

Cyclin-dependent Kinase Inhibitors

The CDKIs are products from various distinct gene families, the prototypes of which are p16 (CDKN2A), and the closely related p21 (CDKN1A) and p27 (CDKN1B).^{16,18} Proteins in the p16 family (p15, p16, p18, p19) are specific inhibitors of CDK4 and CDK6, and their expression and function are dependent on a func-

tional *RB-1* gene product (Rb). Indeed, the absence of Rb appears to make these CDKIs, as well as CDK4 and CDK6, inconsequential in their cell cycle regulatory function. In normal cells, the up-regulation of p16-related proteins is inducible by transforming growth factor- β (TGF- β). The expression of p21 is closely regulated by another unrelated tumor suppressor gene called p53. The p21 gene may be inducible as an effector of growth arrest before S phase entry if the cell must undergo DNA repair. The function of members of the p27 family (p27, p57) appears to be regulated by various signaling pathways that affect cell cycle progression, including those that are mediated by TGF- β or by cyclic AMP.

Regulation of Cyclin-dependent Kinase Activity

Each CDK can associate with multiple cyclins: among the G1 CDKs (CDK2, CDK4, CDK6), CDK2 associates with A, E, and D-type cyclins, whereas CDK4 and CDK6 form complexes only with D-type cyclins. The cyclin partner is required for activation of the kinase, it confers substrate specificity, and it regulates the interactions of the complex with other proteins.

Inactivation of the kinases occurs in part due to the proteolytic degradation of the cyclin partner. CDK activity also is controlled by other kinases and phosphatases. Phosphorylation and dephosphorylation reactions occur at defined residues that are highly conserved throughout the CDK family; for example, phosphorylation of a distal threonine residue (T160/172 in CDK2 and CDK4, respectively) by CAK is an important activating event for all CDKs. Another critical event is dephosphorylation of N-terminal threonine (T14) and tyrosine (Y15/17) residues near the ATP binding site by a bifunctional protein phosphatase of the Cdc25 family. Conversely, phosphorylation of T14 and Y15/17 by the Wee1 bifunctional protein kinase, and dephosphorylation of T161/172 by a protein similar to protein phosphatase 1 are important to inactivate the CDKs. The kinases and phosphatases that carry out this phase of CDK regulation provide additional links between environmental signals and activation of the cell cycle machinery.

MAINTENANCE OF G0

Cell growth and proliferation are integral parts of the immune response. Mature peripheral T cells generally maintain homeostasis in a true resting (G0) state where the cell cycle kinases (CDK1, CDK2, CDK4, or CDK6) and their cyclin partners (cyclin D2, cyclin D3, cyclin E, cyclin A, or cyclin B) are not expressed.¹⁴

Negative Regulation of Cell Cycle Entry

The process of thymic education dictates that naïve T cells must interact with self-peptides presented by MHC (i.e. potential autoantigens) in order to survive.

Naïve T cells express high levels of T cell receptors, so the avidity for self-MHC might trigger their activation and result in autoimmunity. To prevent such activation, several interrelated negative regulatory pathways in lymphocytes enforce quiescence. Regulatory molecules in these pathways include those that dampen signaling such as *Ian5* and negative transcriptional regulators such as the transducer of *ErbB2-1* (*Tob1*), nuclear factor of activated T cells-c2 (*Nfatc2*) and *Smad3*, which is primarily responsible for integrating anti-proliferative and pro-survival signals delivered through the TGF- β receptor.^{11,20} Mutations in these genes result in T cells that survive poorly (*IAN5* knockout), fulminant autoimmunity (TGF- β receptor dominant negative), or cells that show cell cycle dysregulation with eventual tumor formation (*Tob-1* and *NFATc2* knockouts). Intriguingly, the tumor phenotypes for *Tob-1* and *NFATc2* deficiency are B-cell lymphomas, and a recent study suggests that these proteins contribute to this subtype of lymphoma in dogs (A. Frantz and J. Modiano, unpublished data).

THE GO TO G1 TRANSITION AND G1 PHASE PROGRESSION

Antigen binding to the T cell receptor complex triggers a series of events that give rise to a number of interactive, non-linear biochemical cascades that disengage negative regulation and activate the cell cycle machinery. Events during the G1 phase are essential to integrate environmental signals with cellular responses. In some instances, cell proliferation may be separated from other biological responses. For example, stimuli that incite production of interleukins can promote lymphocyte maturation and differentiation, immunoglobulin production, or enhanced cytotoxic or antigen presenting functions and may not lead to cell division. Conversely exogenously provided cytokines may promote proliferation of receptive lymphocytes in a paracrine or endocrine fashion.

Unlike embryonic cells where the length of the cell cycle is temporally fixed, transit time through the G1 phase is variable in adult somatic cells. Potentially transforming events can significantly shorten the G1 phase. In most cases, this prompts activation of checkpoints that prevent transition to the S phase or leads to apoptosis. Cancer cells must, therefore acquire mutations that allow them to overcome these safeguards.¹⁸

The G1 Restriction Point

Cell cycle entry, defined as the transition from G0 to G1, is reversible. This transition point can be defined by partial activation of the CDK4/cyclin D2 holoenzyme complex and phosphorylation of the retinoblastoma susceptibility protein Rb, the product of the *RB-1* prototypical tumor suppressor gene. Transition into G1 also requires activation of CDK6 and CDK2. The CDKI p27 protein may be especially important in control of G1 progression. At least part of the observed increase

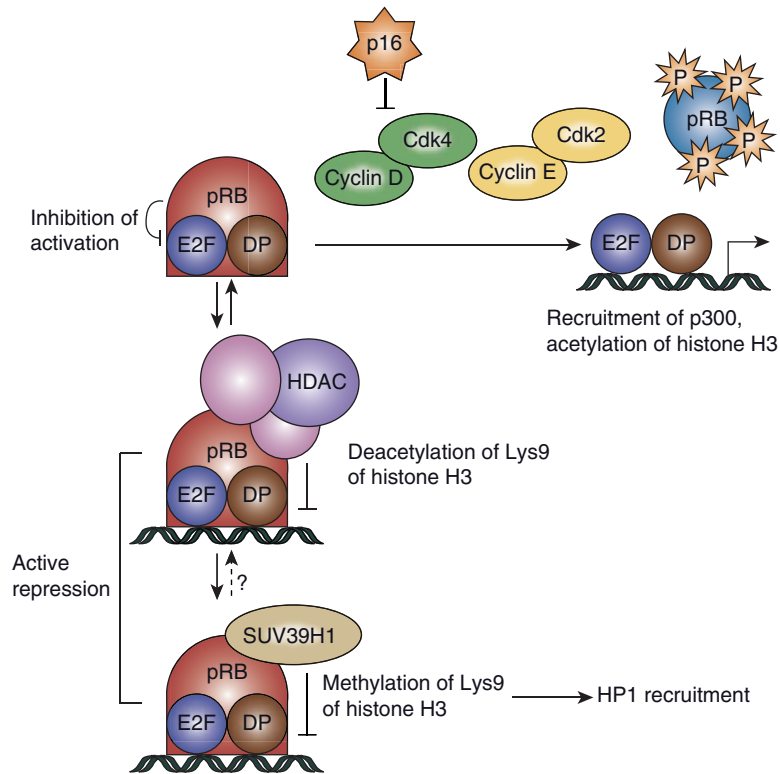


FIGURE 59.2 General mechanisms that control the repressive activity of E2F. The retinoblastoma protein binds to an E2F–DP complex in G0/G1 cells and this leads to repression of E2F-responsive genes through two possible mechanisms. First, phosphorylated RB (pRb) inhibits E2F from activating transcription by binding to its transactivation domain and preventing its interaction with the core transcriptional machinery. Second, the resulting complex binds to the promoters of E2F-responsive genes and enforces their “active repression” through recruitment of either histone deacetylases (HDACs), which remove the acetyl group from lysine 9 (Lys9) of the histone H3 tail and thereby facilitate nucleosome packing, or SUV39H1, which methylates the same lysine residue to create binding sites for HP1, leading to transcriptional silencing. Cell-cycle entry is dependent on the sequential activation of the cell-cycle-dependent kinases, CDK4/6–cyclin D and CDK2–cyclin E, which phosphorylate pRB and cause it to release E2F. The resultant activation of E2F-responsive genes seems to be at least partially dependent on the ability of E2F to recruit p300, leading to the acetylation of Lys9 of histone H3. Several key components of the pRB regulatory pathway are known to be tumor suppressors (pRB, p16) or oncogenes (cyclin D, Cdk4). DP, differentiation regulated transcription factor (DRTF) protein; pRB, retinoblastoma protein. (Reproduced from Trimarchi JM, Lees JA, Sibling rivalry in the E2F family. *Nat Rev Mol Cell Biol* 2002;3:11–20, with permission. ©Macmillan.)

in CDK2 activity in activated T cells may be due to IL-2-induced dissociation of p27 from CDK2 kinase complexes, and T cells from p27-knockout mice have exaggerated T cell responses.⁵ Cells that reach this stage of activation acquire the capability to respond to proliferative signals, but are not committed to continue transit into the S phase until they cross the restriction point, R.¹⁴ This occurs at least in part by activation of CDK4/cyclin D3 and CDK2/cyclin E complexes.

Regulation of the G1 to S Transition by Rb

The so called pocket proteins of the Rb family is a group of proteins that promote (or suppress) the expression of genes that control the G1/S transition, including members of the E2F family of transcription factors and histone deacetylases (HDACs). In the hypophosphorylated state, modulation of these proteins by Rb acts as a cell cycle brake. Phosphorylation of Rb by the CDKs leads to functional collapse of the pocket, releasing latent transcription factors and allowing their activa-

tion. It appears that phosphorylation by CDK4 releases HDACs, relieving transcriptional repression for some genes such as cyclin E, and opens the conformation of Rb, making it permissive for phosphorylation by CDK2. Subsequent Rb phosphorylation by CDK2/cyclin E releases E2F transcription factors. The activation of E2F factors requires their association with DP-1 or DP-2 to form heterodimers that orchestrate transcription of genes necessary for DNA synthesis (Fig. 59.2). In lymphocytes, activation of Aurora B in conjunction with Survivin and mTOR is an essential step to complete the transition from G1 to the S phase.

S PHASE PROGRESSION

In mammalian cells, an important control element in the process of DNA replication is assembly and activation of pre-replication complexes at 20,000–40,000 genetic sites when a complete single copy of DNA complement is made. Initiation sites (the replication origin) interact

with a group of proteins called the Origin of Replication Complex (ORC).¹⁹ This allows helicases and other enzymes to unwind the double strand and recruit polymerases. Replication forks then move away from the origin in either direction (Fig. 59.1).

As is true with abnormal G1 transit, partial re-replication triggers checkpoints that arrest cell cycle progression or promote apoptosis. Re-replication is in some ways unavoidable and may be a driver of evolution. Indeed, it is now clear that subtle differences in DNA copy number exist among individuals within a species, and in dogs these might be conserved within members of the same breed, similarly to microsatellites and single nucleotide polymorphisms.^{3,12}

Checkpoint Transit

The S-phase checkpoint relies on detection of stalled replication forks (usually due to insufficient trinucleotide building blocks) or DNA damage. Members of the ATM/ATR kinase families (ataxia-telangiectasia mutated-and Rad3 related) act as sensor kinases, which in turn activate the effector kinase Chk2. Chk2 activation delays or arrests S phase progression. Persistent stalled forks or DNA damage that cannot be repaired triggers apoptosis of normal cells.

Regulation of the Origin or Replication

Cells must avoid extensive re-replication during DNA synthesis. The ORC contains six stable subunits that bind to DNA in the transition from mitosis to G1 (or G0), followed by additional proteins, such as MCM helicases, that are synthesized or stabilized at or near the G1 to S transition to form the pre-replicative complexes. CDK2/cyclin E (and other S phase kinases) primes these pre-replicative complexes to form pre-initiation complexes that are necessary for activation of DNA polymerases. Progression through S phase requires transition from CDK2/cyclin E to CDK2/cyclin A complexes, which are followed by the appearance of CDK1 complexed to cyclin A and cyclin B. Substrate specificity is probably important to prevent re-replication and to maintain the fidelity of DNA polymerases.

G2 PHASE PROGRESSION

Transition from S to the G2 phase occurs upon completion of DNA replication. At this point, somatic cells are tetraploid (i.e. have four copies of DNA) and are destined to divide.

DNA Damage Checkpoint

The S phase checkpoint is fallible, so a partially redundant checkpoint exists during the G2 phase. ATM/ATR kinases also act as G2-DNA damage sensors. Lymphocytes that undergo physiologic DNA double strand breaks during antigen receptor gene rearrange-

ment are particularly susceptible to chromosomal instability, which is why patients with ataxia telangiectasia have hypofunctional adaptive immune responses and a significantly higher risk of developing cancer.² The primary G2 DNA damage effector kinase is Chk1, which controls G2 phase progression and M phase entry, primarily by phosphorylating the CDK-regulatory CDC25 phosphatases.

Preparation for Mitosis

Proper chromatid alignment and association is necessary for normal separation during mitosis. Spindle assembly and the spindle checkpoint are regulated by CDK1 in conjunction with the Aurora A and Aurora B kinases, respectively. Aurora C kinase controls chromosomal segregation in mitosis.

M PHASE PROGRESSION

Chromatin condensation occurs throughout the cell cycle until the onset of mitosis, when the chromatin is packaged into species-specific units called chromosomes. M phase progression begins with the dissolution of the nuclear membrane, continues with the assembly of the mitotic spindle, and culminates with division into two daughter cells, each with a diploid complement of DNA.

Prophase

Breakdown of the nuclear envelope proceeds at the end stage of chromosome condensation by phosphorylation of lamins. This precedes spindle formation, which is mediated by complex interactions of microtubules and kinetochores. Centromeres allow chromosomes to attach to the spindle, a process that also may be controlled through Aurora B kinase and Survivin.¹

Metaphase

As cells approach metaphase, chromosomes align along the spindle at equal distance from each pole to form the traditional metaphase plate in a process called congression.

Anaphase

Cyclin B is rapidly degraded by the anaphase promoting complex (APC), which functions as an E3 ubiquitin ligase; this is followed by separation of sister chromatids. Anaphase promoting complex activity is restrained by the spindle checkpoint until kinetochore alignment is complete. Sister chromatid separation is itself a complex process, and is controlled at least in part by DNA topoisomerase II. The importance of this enzyme in mitosis is underscored by the exacting sensitivity of cancer cells to camptothecin and other topoisomerase II inhibitors.

After the separation of sister chromatids, there is little resistance for chromosome segregation towards the poles. Vesicles on the chromosomes promote reformation of the nuclear envelope and cytokinesis ensues as CDK1/cyclin B activity subsides. Each daughter cell can then resume its normal function or re-enter the cell cycle.

THE CELL CYCLE AND CANCER

Cancer is a condition where cells divide uncontrollably, making it easy to see how it can be linked to abnormal cell cycle function. Common abnormalities include inactivation of CDKs through mutation or deletion, inappropriate expression of D-type cyclins, inactivation of Rb, and malfunction of DNA damage checkpoints, among others.⁹ Not surprisingly, these gross abnormalities are prognostic, but even subtle quantitative differences in cyclin expression, Rb phosphorylation, etc., can influence tumor progression and serve as useful indicators of prognosis for hematopoietic tumors.¹³ Indeed, recent unpublished data from our laboratory suggest that gene expression signatures that are indicative of aberrant cell cycle regulation are hallmarks of canine high-grade B cell malignancies and canine osteosarcomas that respond poorly to conventional therapy (K. Gavin, A. Frantz, A. Sarver, T. Phang, and J. Modiano, unpublished observations).

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Epidemiology of Hematopoietic Neoplasia

MICHELLE G. RITT

Hematopoietic Neoplasia Epidemiology based on a Reference Population
 Reference Population Information for Dogs
 Reference Population Information for Cats
 Reference Population Information for Horses
 Reference Population Information for Cattle
 Hematopoietic Neoplasia Risk Assessment in Dogs
 Lymphoma
 Environmental risk factors
 Genetic/heritable risks
 Geographic risk factors
 Leukemia
 Histiocytic Sarcomas
 Plasma Cell Tumors

Mast Cell Tumors
 Hemangiosarcomas
 Hematopoietic Neoplasia Risk Assessment in Cats
 Lymphoma/Leukemia
 Environmental
 Infectious diseases
 Genetic/heritable
 Mast Cell Tumors
 Hemangiosarcoma
 Hematopoietic Neoplasia Risk Assessment in Horses
 Hematopoietic Neoplasia Risk Assessment in Cattle
 Lymphoma
 Conclusions

Acronyms and Abbreviations

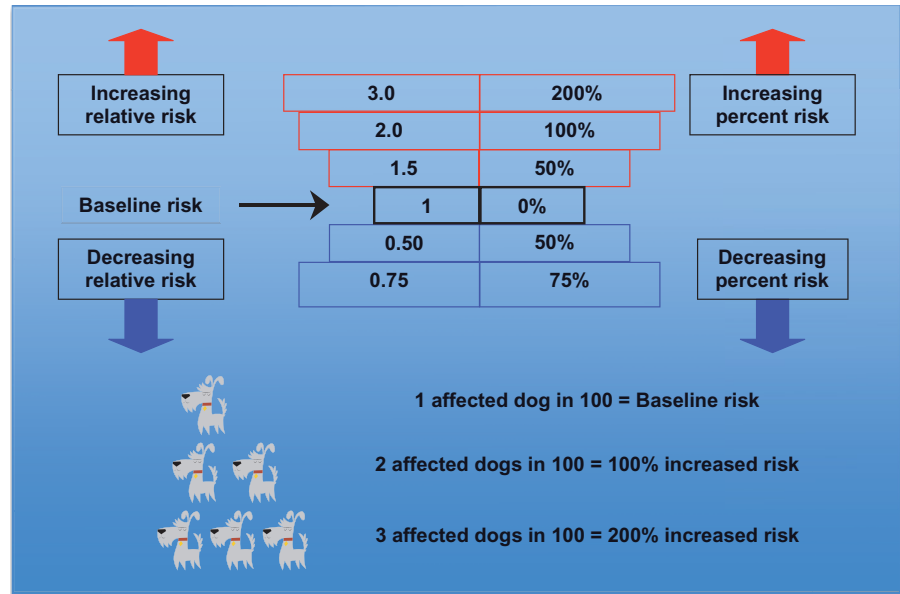
2,4-D, 2,4-dichlorophenoxyacetic acid; BLV, bovine leukemia virus; FeLV, feline leukemia virus; FIV, feline immunodeficiency virus; VDL, veterinary diagnostic laboratory.

The simplest definition of epidemiology is the study of factors which define a population's risk of susceptibility to a given disease. Both genetic and environmental influences are usually important, particularly when identifying risk factors for neoplasia. Epidemiology data have inherent uncertainty, as they seek to identify the probability that disease will develop within a given population or the risk of one population to develop a particular disease as compared to another population without that risk. The predominant statistical terms used in cancer epidemiology are relative risk and absolute risk. Relative risk is the comparison between two groups: one group has a given risk factor and the other group does not. Absolute risk is the probability that cancer will develop within a defined period of time. If the period of time is from birth to death it is referred to as lifetime risk (Fig. 60.1). In order to establish risk factors, data from a test population are compared to a reference population. Replication of the data is required for validation. The ultimate goal is to have the most representative group to which other popula-

tions can be objectively compared. There are vast amounts of information of this type available for neoplasia in human beings; however, it is significantly lacking for other species.

Epidemiology of hematopoietic neoplasia in domestic animals is an understudied area. By far, the most information available is for dogs. Yet even in dogs, the most current database regarding a reference population is 40 years old. The reference population itself has changed considerably and needs to be updated to reflect: (1) breed popularity changes; (2) changes in breeding practices to produce different desirable traits; (3) changes in production management and husbandry practices; (4) popular sire effects; (5) development of new breeds, and breeds from narrow gene pools; (6) geographical changes; and (7) changing infectious disease influences. More recent studies generally have involved significantly smaller numbers of animals and have not been based on reference populations. This means many specific risk factors will be difficult to identify.

FIGURE 60.1 Two different ways of looking at cancer risk. In the upper portion of the figure, baseline relative risk is equivalent to 1 or 0% risk in the population. When risk increases to 50%, relative risk increases by 1.5× the baseline. When the overall risk decreases by 50%, relative risk decreases to 0.5× the baseline. In the bottom portion of the figure, this is represented by the example of a baseline risk of one affected dog in a given population of 100 dogs: when two dogs are affected, the risk increases by 100%; when three dogs are affected, the risk increases by 200%.



HEMATOPOIETIC NEOPLASIA EPIDEMIOLOGY BASED ON A REFERENCE POPULATION

A pioneering study on cancer epidemiology in dogs and cats was the first of its kind to investigate the implications of sex and age in a defined population in 1968.⁴ Although the data encompassed a limited geographical region, this paper was among the first to describe a higher incidence of cancer in purebred dogs compared to mixed breeds. Above all, this paper underscored the need for a more geographically comprehensive reference regarding the susceptibilities and risks of domestic animals for developing neoplasia. A much broader study and the only animal cancer reference population still available today comes from a compilation of data from 15 veterinary schools in the USA and Canada through the years 1964–1977.²² The geographical bias is somewhat minimized, however, by comparing animals from schools from a diverse group of geographic locations.

Reference Population Information for Dogs

In dogs with hematopoietic neoplasms, lymphoma represented the most cases (1,324), followed distantly by hemangiosarcoma of the spleen (referred to as angiosarcoma) with 165 cases, and leukemia (subclassifications not defined) at 151 cases. Breeds that represented the highest risk for lymphoma, leukemia, and hemangiosarcoma (spleen) included Boxers, Basset hounds, and St. Bernards, which were 3–4 times as likely as all breeds combined (reference population) to develop these cancers. Breeds twice as likely to develop lymphoma, hemangiosarcoma, and leukemia included Scottish terriers, Bulldogs, Airedales, Weimaraners, golden retrievers, Doberman pinschers, Labrador retrievers, English

setters, and Great Danes. Dogs considered less likely to develop these tumors (relative risk < 1) were mixed breeds, miniature and toy poodles, Pomeranians, Chihuahuas, Boston terriers, Cocker spaniels, and dachshunds.

When hemangiosarcoma was evaluated separately from leukemia and lymphoma, breeds with the highest risk (2–3 times greater) included: Boxers, Great Danes, German shepherd dogs, English setters, golden retrievers, pointers and German shorthaired pointers. Breeds with decreased risk included miniature schnauzers, Brittany spaniels, Cocker spaniels, Beagles, Boston terriers, miniature and toy poodles, dachshunds, and Pekingese, with Chihuahuas showing the lowest risk.

There were 202 cases of mast cell tumors in the skin and 162 cases of cutaneous hemangiosarcoma in the database. The relative risk was similar to hematopoietic and lymphatic neoplasms; however, spayed females were 1.3 times more likely to develop these tumors. Also, it should be noted that the incidence of mast cell tumors was only reported in dogs.

Hematopoietic tumors not considered in this study included plasma cell tumors and histiocytic sarcomas (with the exception of histiocytomas which were considered along with benign neoplasia). Histiocytomas were by far more prevalent in dogs than cats.

An independent study that reviewed the database of the Veterinary Medical Data Program from 1964 to 1989 to determine the incidence of neoplasia in dogs of 6 months of age or younger showed that cancers of the hematopoietic system were most frequent in this population.¹³

Reference Population Information for Cats

Lymphoma was reported in 657 cats. Manx cats were 4.6 times more likely than non-Manx cats, and Burmese

were 3.1 times more likely than non-Burmese to develop lymphoma. Females, neutered and intact, had slightly lower relative risk than males. Four hundred and seventeen cases of leukemia were identified. Mixed breed cats were 1.3 times as likely to develop leukemia as purebred cats, and Persians were 1.2 times as likely as non-Persians to develop leukemia. Siamese cats had a lower relative risk than the average for the reference population. Feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV) status were not evaluated.

Reference Population Information for Horses

Forty-eight cases of equine lymphoma were recorded, as were five cases of leukemia. Appaloosas, mixed breeds, and Standardbreds were 1.7, 1.5, and 1.2 times as likely to develop lymphoma respectively. Mares were 1.6 times as likely as geldings and stallions to develop lymphoma.

Reference Population Information for Cattle

There were 332 cases of bovine lymphoma and 56 cases of bovine leukemia recorded in the database. Gender was found to be the most significant risk factor. Steers were 3.8 times as likely as bulls, and cows were twice as likely as bulls to develop lymphoma. Bovine leukemia virus (BLV) status was not evaluated. Breeds at highest risk for lymphoma included Angus and Ayrshire (1.6×), Jerseys (1.5×), mixed breed dairy and shorthorns (1.3×), mixed breed beef and Holsteins (1.2×) while Guernseys, Herefords, and Charolais had lower relative risk.

HEMATOPOIETIC NEOPLASIA RISK ASSESSMENT IN DOGS

Lymphoma

Environmental Risk Factors

Because it is one of the most common malignant neoplasms affecting domestic dogs, lymphoma has been very extensively studied in dogs (see Chapters 62, 67, and 70). Many attempts have been made to associate lymphoma with environmental risk factors; however, none has been conclusively verified. One environmental risk factor evaluated was exposure to the lawn herbicide 2,4-dichlorophenoxyacetic acid (2,4-D). Although one study found a relationship, a subsequent re-evaluation was unable to confirm the association.^{10,12} An association between exposure to electromagnetic fields and the subsequent development of lymphoma in dogs was published but follow-up studies have not been performed to validate that connection.²⁴

A study retrospectively performed in the Tuscany region of Italy evaluated dogs with residential exposure to environmental pollutants from 1996 to 1998.⁶ The results of this study suggested that there might be an

increased risk of lymphoma in dogs living in or near industrial areas and dogs that may have had environmental exposure to household paints and solvents. However, the study's findings were subject to bias recall on the part of the dogs' owners (information obtained by questionnaires and telephone interviews), and specific information regarding types of chemical exposure and amount of exposure were not investigated. The authors of this study affirm that additional investigation is warranted to validate these potential associations.

Genetic/Heritable Risks

It has been postulated that perhaps no other species shows more variability and flexibility of its genome than the domestic dog.²⁰ *Canis familiaris* has, through selective breeding practices by humans, had its gene pool manipulated to produce more than 400 distinct breeds ranging in size from 2 kg to over 100 kg. Life expectancy, hair coat, skull and skeletal conformation, and behaviors all have been diversified in just 2,800 years.²⁰ There is abundant evidence that humans also have altered dogs' susceptibility to cancer. Epidemiologic evidence for genetic risk factors for lymphoma in domestic dogs is currently limited to breed associations. The occurrence of tumors is sporadic; however, their development is affected to an extent by heritability. The prevalence of lymphoid neoplasms in dogs differs among breeds as does the frequency of B cell and T cell neoplasms; however, heritable risk factors have not been identified.

More recent information regarding hematopoietic cancers in dogs can be obtained from health surveys privately commissioned by specific American Kennel Club National Breed Clubs. This information is collected via voluntary recruitment of members of the national breed clubs who complete a written survey. The data are then compiled by statisticians and are presented as a health report for the specific breed club. These surveys are not normalized to a reference population and may contain considerable bias. Diagnoses are unable to be directly confirmed by the investigators and sometimes by a veterinarian so there is considerable variation in the numbers of responses obtained. Nevertheless, taken within the context of their limitations, breed health surveys can provide further insight into the epidemiology of hematopoietic neoplasms in dogs.

Based on analysis of data for golden retrievers, Irish setters, Rottweilers, Portuguese water dogs, English Cocker spaniels, and Gordon setters, the risks for hematopoietic neoplasia were combined to include lymphoma, mast cell tumors, and hemangiosarcoma.^{7,8,27-30} A meta-analysis of the available data, using the chi square test, indicates golden retrievers have the highest risk of the three hematopoietic tumors combined, followed distantly by Gordon setters, which, in turn were at higher risk than Portuguese water dogs, Rottweilers, and Irish setters.²⁷⁻³⁰ The last three breeds were roughly equivalent in prevalence for these hematopoietic

neoplasms. When the prevalence of lymphoma was considered separately from mast cell tumors and hemangiosarcoma, the rank order of apparent breed risk did not change. When a non-hematopoietic neoplasia such as osteosarcoma was evaluated using the same breed survey information, the rank order of breed risk changed considerably. Irish setters had the highest absolute number of affected dogs followed by golden retrievers, Rottweilers, Gordon setters, and, more distantly by Portuguese water dogs. English Cocker spaniels had an insignificant risk for osteosarcoma.

Geographic Risk Factors

There may be geographic risk factors; however, data are regionalized and sporadic. For example, Jagielski et al. found in a study of dogs admitted to the Warsaw Veterinary College between 1998 and 2000 that the odds ratio for Rottweilers to be diagnosed with multicentric lymphoma was 6-fold greater than dogs from other breeds.¹¹ However, the study populations may not accurately reflect the general dog population in Poland or elsewhere. This information certainly is a valuable starting point for more thorough and controlled epidemiological studies; however, one must guard against over-interpretation of this type of data.

Similarly, a study comparing the lifetime occurrence of neoplasia in German Shepherd dogs and Belgian Malinois military working dogs (United States Department of Defense) during the year 1992 reported that more Belgian Malinois were diagnosed with lymphoma than were German Shepherd dogs and that more German Shepherd dogs were diagnosed with hemangiosarcoma.²¹ The authors remind the readers that the study's limitations include that these dogs spend a proportion of their lives outside of the USA in various regions around the world, and that these dogs have a standard high nutritional plane and level of fitness as well as handlers that are extremely vigilant over their welfare. Thus, these groups are being evaluated as they relate to each other and the study's findings should not necessarily be generalized to the Belgian Malinois and German Shepherd dog breeds as a whole.

Investigators in Austria⁹ classified subtypes of canine lymphoma and then compared their results with findings in other countries in Western Europe and found no difference in morphology and immunophenotype of lymphomas. They concluded from this information that Austrian dogs are unlikely to have significantly different risk factors than other canine populations in Western Europe.

Leukemia

Leukemia appears to be less common than lymphoma in the canine population and, although leukemia may be under diagnosed, there is no new information on the specific epidemiology of this neoplasm since the study by Priester and McKay (see Chapters 63 and 65).²²

Histiocytic Sarcomas

Cutaneous histiocytomas are associated with young dogs (<6 years of age) and are typically benign in nature. Breeds historically associated with a higher incidence of cutaneous histiocytoma include Boxer dogs and Bulldogs. Scottish terriers, Doberman pinschers, and Cocker spaniels also have been suggested as breeds with higher risk to develop this neoplasm.⁵

The malignant counterparts of cutaneous histiocytic sarcoma are collectively known as the histiocytic sarcoma complex, which comprises a spectrum of malignancy rather than a static neoplastic process (see Chapter 71). The histiocytic sarcoma complex includes both localized and disseminated forms. Breeds at highest risk are reported to be Bernese mountain dogs, golden and flat coated retrievers, and Rottweilers.⁵ It should be noted that histiocytic sarcomas are not related to malignant fibrous histiocytomas, which are classified with the tumor group of soft tissue sarcoma.

Plasma Cell Tumors

An increased frequency of lingual plasma cell tumors was found in Cocker spaniels.³ This study evaluated 1196 cases of lingual lesions that were submitted to the Colorado State University Veterinary Diagnostic Laboratory during the years 1995–2004. Previously, Cocker spaniels were suspected of an increased risk to develop mucocutaneous plasmacytomas in a study of 75 cases published in 1989.²³

Mast Cell Tumors

Mast cell tumors can occur in any breed of dog and mixed breed dogs; however, there have been associations made with specific dog breeds that seem to have increased susceptibility (see Chapter 66). A study from Sydney, Australia considering data from 1989 to 2001 at the University Veterinary Center found that Boxers, Australian cattle dogs, and Staffordshire bull terriers were at a significantly higher risk for mast cell tumors.¹ Pugs, Boston terriers, and other brachycephalic breeds also have been present in statistically significant numbers in other studies.

Hemangiosarcomas

Hemangiosarcoma has recently been suggested to originate from progenitor cells in the bone marrow rather than from endothelial lining cells of the organs in which the solid tumors have originated (see Chapter 72).¹⁵ Recent evidence suggests that these tumors may merit reclassification from the soft tissue sarcoma group into the hematopoietic neoplasms. Interestingly, Priester and McKay also categorized lymphoma, leukemia, and hemangiosarcoma together in their publication in the late 1970s.²²

Hemangiosarcoma is more common in dogs than any other species. Data from six consecutive years were used to evaluate the prevalence of canine hemangiosa-

roma. Two hundred and twenty two canine samples of nonvisceral hemangiosarcoma (mostly cutaneous) received by the Veterinary Diagnostic Laboratory (VDL) at Colorado State University in 1996, 1997, and 2002, revealed equal numbers of male and female dogs affected. The most common breeds, when corrected for popularity bias, included pit bull terriers (most common), and sight hounds (whippets, greyhounds, and Italian greyhounds, specifically). The largest number of submissions came from golden retrievers and German Shepherd dogs. Lingual hemangiosarcoma risk was shown to be increased in Border collies while Beagles were shown to have an increased risk for concurrent lingual and cutaneous hemangiosarcoma.³ Three hundred and eight tissue samples of canine visceral hemangiosarcoma evaluated exclusively from year 6 of the VDL study showed an approximately equal sex distribution. However, the most common breeds affected were golden retriever (56 cases), Labrador retrievers (45 cases) and then German Shepherd dogs (30 cases).²⁵

Srebernik and Appleby³¹ reported a retrospective study of 4,535 cases of canine vascular tumors evaluated from 1968 to 1989 at the Royal Veterinary College (UK). This study showed a significantly higher risk among German Shepherd dogs for hemangiomas and hemangiosarcomas with roughly 66% of those cases being hemangiosarcomas. They also found that the spleen was the most frequent site in German Shepherd dogs with males being overrepresented. German Shepherd dogs had a lower frequency of cutaneous and subcutaneous hemangioma and hemangiosarcoma.

HEMATOPOIETIC NEOPLASIA RISK ASSESSMENT IN CATS

Lymphoma/Leukemia (Lymphoid Neoplasms)

Environmental

A recent questionnaire-based study suggested that cats with exposure of 5 years or longer to secondhand household tobacco smoke were 3.2 times more likely to develop lymphoma than cats from nonsmoking households.² Although some recall bias is inevitable in this type of study, it is more limited here as the study focused on only one parameter of which the owners would have a first hand knowledge. Nevertheless, this study needs to be further validated.

Infectious Diseases

Feline retroviruses as risk factors have had a significant impact on the development of lymphoid neoplasms in the cat. The so called "Feline leukemia virus infection era" comprises the late 1970s through 1983.¹⁷ This period of time showed a high incidence of lymphoma in particular associated with FeLV infection. A retrospective study from 1968–1988 of 53 cats from the Pacific Northwest region with matched controls showed that

cats infected with the FeLV virus had a 62.1 times greater risk of developing leukemia or lymphoma than cats that were uninfected.²⁶ Cats infected with both FeLV and FIV had an increased risk of 77.3 times greater than the control population for these neoplasms, and cats infected with FIV alone had a 5.6 times greater risk than uninfected cats.²⁶ After 1983, the impact of vaccination against FeLV and culling of infected animals determined by FeLV testing resulted in a significant reduction in FeLV infection. Cases of non-FeLV associated lymphoma (i.e. thymic and alimentary lymphomas) began to rise in the feline population.²⁶

Genetic/Heritable

Siamese cats and other related Oriental breeds have demonstrated an increased risk for development of mediastinal lymphoma at younger than 4 years of age in multiple studies.^{17,26} Some cats were infected with FeLV.²⁶ Nevertheless, this information indicates a potential shift in breed risk in feline lymphoma compared to the reference population evaluated 40 years ago.²² In that population, Siamese cats had a lower than average risk of developing lymphoma.

Mast Cell Tumors

Siamese cats are predisposed to mast cell tumors compared with other breeds.¹⁹ In an assessment of 41 cats with mast cell tumors published in 2006, young Siamese cats were shown to be more likely to present with a military histiocytic form of cutaneous mast cell tumors.¹⁶ It appears that visceral mast cell tumors (majority in the spleen) are more common in cats. Currently, there is no known association of infectious disease with mast cell tumor development.

Hemangiosarcoma

Hemangiosarcoma continues to be a rare tumor in cats and no specific risk factors have yet been identified in this species. Evaluation of 72 histopathological samples of hemangiosarcomas in cats submitted to the VDL at Colorado State University over a 6 year period showed that the majority of these tumors were cutaneous (59 out of 72), there was no noted sex predilection, and the average cat age was 10 years.²⁵

HEMATOPOIETIC NEOPLASIA RISK ASSESSMENT IN HORSES

The only current information on epidemiology of lymphoma suggests that T cell multicentric lymphomas are more common than B cell multicentric lymphomas in horses.¹⁸ Hemangiosarcoma in horses is rare and no specific risk factors have yet been identified. A total of 11 samples of equine hemangiosarcoma were identified in the previously mentioned study by Schultheiss through the VDL at Colorado State University in a 6-year period.²⁵

HEMATOPOIETIC NEOPLASIA RISK ASSESSMENT IN CATTLE

Lymphoma

The only hematopoietic neoplasia of clinical importance in cows, based on the literature to date, is lymphoma. One risk factor identified in cows for lymphoma is infection with bovine leukemia virus (BLV). It is estimated that 2–5% of BLV cows develop lymphoma, representing about 5,200 cows/year in the state of California alone.¹⁴

CONCLUSIONS

The paucity of specific data based on an updated reference population regarding hematopoietic neoplasia in domestic animals underscores the need to define specific risk factors for these diseases. By linking large databases with updated communication technology, and by addressing factors affecting populations of domestic animals that have relevance to development of hematopoietic cancer, more accurate perceptions of risk can be ascertained. Changes in breeding practices and husbandry based on better-defined risk factors can then be implemented to help reduce the incidence of hematopoietic neoplasia for those animals that are significantly threatened. In the big picture, by deepening our understanding of susceptibility to cancer we also come to understand its nature more profoundly which will lead us to earlier diagnosis and, ultimately, more effective therapy.

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Genetics of Hematopoietic Neoplasia

JAIME F. MODIANO and MATTHEW BREEN

Genes and Cancer Risk

- Heritable Cancer Syndromes
- Genetic Influence in Sporadic Cancers

The Hallmarks of Cancer

- Self-sufficiency of Growth Signals
- Insensitivity to Anti-growth Signals
- Evasion of Apoptosis
- Limitless Replicative Potential

Sustained Angiogenesis

- Invasion and Metastasis

Molecular Events in Carcinogenesis

- Initiation, Promotion, Progression
- Genetic Instability
- Epigenetic Events
- Cancer Stem Cells
- Adaptive Evolution in the Tumor Microenvironment

Acronyms and Abbreviations

DNA, deoxyribonucleic acid; HIF, hypoxia inducible factor; IL, interleukin; LOH, loss of heterozygosity; LTR, long terminal repeat; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; RCND, renal carcinoma and nodular dermatofibrosis; VEGF, vascular endothelial growth factor-A; VEGFR, vascular endothelial growth factor receptor; VHL, von Hippel-Lindau (tumor suppressor gene).

It is now clear that cancer is a disease driven by the accumulation of genetic abnormalities.³⁰ Hundreds of genetic abnormalities that are peculiarly associated with specific cancers have been identified. In fact, technological advances have allowed full sequencing of tumor genomes.¹⁷ The information from these experiments has reinforced current concepts and provided insight into new areas of research. This chapter will focus on how interactions between genes and environment impact the origin, progression, and response to therapy of hematopoietic tumors.

GENES AND CANCER RISK

To understand cancer, one must first realize this is neither a single nor a simple disease. Rather, the term *cancer* describes a large number of diseases whose only common feature is uncontrolled cell growth and proliferation. An important concept that is universally accepted is that cancer is a genetic disease, although it is not always heritable. Tumors arise from the accumulation of mutations that eliminate normal constraints of proliferation and genetic integrity in a somatic cell.

Among other causes, mutations can arise due to the inherent error rate of enzymes that control DNA replication, which introduces from 1 in 10 million to 1 in a million mutations for each base that is replicated during each round of cell division. Most mammalian genomes comprise 2–3 billion base pairs, so every time a cell divides, each daughter cell is likely to carry at least a few hundred mutations in its DNA. Most of these mutations are silent; that is, they do not hinder the cell's ability to function. However, others can disable tumor suppressor genes or activate proto-oncogenes that respectively inhibit or promote cell division and survival.

Heritable Cancer Syndromes

The existence of genetic predisposition to cancer is illustrated by well-defined heritable cancer syndromes.⁶ Even though they account for fewer than 5% of all human cancers, studies of families with these syndromes provided many of the initial clues to understand the genetic basis of sporadic (non-heritable) cancers. Although inheritance is recessive, these familial cancer syndromes show dominant patterns of inheritance and have high penetrance. All but two of the

known familial cancer syndromes are due to mutations that inactivate tumor suppressor genes. As originally proposed by Knudson in his “two-hit” hypothesis from studies in children with retinoblastoma,¹³ individuals at risk are obligate heterozygotes (they inherit a mutant allele and a wild type allele). As it happens, homozygous mutations in critical growth regulatory genes usually cause embryonic lethality; but in the case where a single allele is affected, the mutation is present in every cell in the body. Given the rate of spontaneous mutation described above, the probability that the second, wild type allele will be inactivated in at least one cell is extremely high, therefore facilitating tumorigenesis. This process is called loss of heterozygosity (LOH).

A mutation in a single gene can predispose individuals to distinct cancer syndromes, while independent, single mutations of different genes can result in virtually the same disease.⁶ This is not surprising when we consider that commonly affected genes are multifunctional and are parts of complex interactive networks or circuits, so a mutation may only alter gene function along one biochemical pathway, leaving its interactions with other pathways intact. Moreover, mutations that contribute to most sporadic cancers are restricted to a small subset of genes, many of which also are associated with heritable cancer syndromes.

At least one heritable cancer syndrome (renal carcinoma and nodular dermatofibrosis; RCND) has been described in German Shepherd dogs.¹⁹ The heritable factor (or *RCND* gene) for this syndrome maps to dog chromosome 5 (CFA 5), and specifically to the folliculin gene, which was recently described as corresponding to the human disease (Birt-Hogg-Dube syndrome).²⁶

Genetic Influence in Sporadic Cancers

Both the environment and the individual’s peculiar genetic background influence cancer risk and the natural history of tumors. This is especially clear in mice, where the relative rate of spontaneous cancers and the susceptibility to chemically induced cancers differ according to the genetic background of various inbred strains. Similar evidence exists for humans; for example, the risk of habitual smokers to develop lung cancer is tightly linked to a unique allele encoding the alpha-3 subunit of the high affinity nicotinic receptor.¹ There also is evidence to suggest that in some cases mutations are “directed” due to the presence of a “mutator phenotype,” where the factors that control DNA replication and repair are prone to more errors than would be expected by simple random events. This leads to different rates of cancer predisposition, which would be higher than the mean in individuals bearing this mutator phenotype, and might explain why not all people or animals exposed to similar environmental carcinogens develop the same forms cancer at the same rate.²⁰

In dogs and other domestic animals, the co-existence of genetic isolates in closed populations we call breeds, along with animals of mixed breeding, lends itself to study how a relatively homogeneous background influ-

ences cancer in outbred populations. Preliminary data from whole genome association studies suggest there are distinct heritable traits that segregate with common cancer phenotypes in dogs.^{18,29} Some of these traits are shared between closely related breeds, whereas others seem to contribute to risk independently in different breeds. Perhaps more importantly, dogs are the first species where genetic background has been shown to mold tumor genomes and tumor gene expression profiles.^{22,37,38} Together with the demonstration that causal, pathognomonic genetic abnormalities are conserved in homologous human and canine cancers, this opens up a new area to identify the precise contribution of heritable traits to sporadic cancers using comparative systems approaches.

THE HALLMARKS OF CANCER

Six essential, acquired characteristics are necessary for cellular transformation (Fig. 61.1).⁹ These characteristics, are: (1) self-sufficiency in growth signal; (2) insensitivity to anti-growth signals; (3) the ability to evade apoptosis; (4) limitless replicative potential; (5) sustained angiogenesis; and (6) the capacity to invade tissues and metastasize. Some of the important concepts

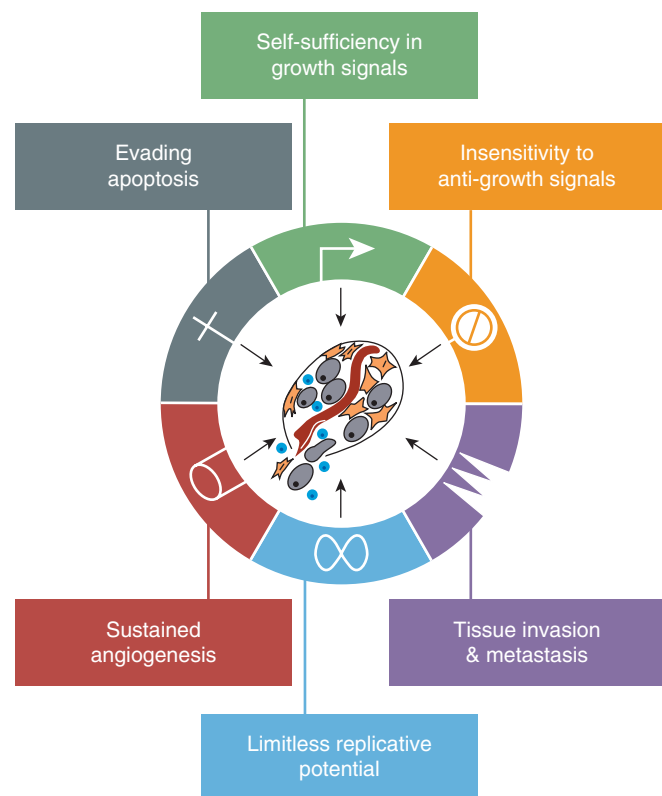


FIGURE 61.1 Acquired capabilities of cancer. Most if not all cancers acquire the same set of functional capabilities during their development, albeit through multiple mechanistic strategies. (Reproduced from Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57–70, with permission. ©Elsevier.)

that are clarified by this list include: no single gene is universally responsible for transformation; five or six mutations are the minimum probable number required to endow the cancer phenotype; each step is regulated by multiple interactive biochemical pathways, thus, mutations of different genes along a pathway can result in equivalent phenotypes and conversely, mutations of the same gene can result in different cancers with distinct biology; tumors behave as tissues, and the interactions between the tumor and its microenvironment are major drivers of cancer behavior.

Self-Sufficiency of Growth Signals

Cells communicate with each other and integrate environmental signals by sensing cues and gradients. For example, migration, metabolism, and proliferation of mature hematopoietic cells are regulated in autocrine and paracrine fashions by locally secreted cytokines. The same cytokines may travel systemically and act in an endocrine fashion. Generally, the cytokines work by binding transmembrane receptors, which in turn initiate signaling cascades that culminate in transcriptional changes. The activity of these cytokines, their receptors, and the corresponding signaling molecules are finely tuned. The system can be shut down when the concentration of the cytokine falls below a threshold that can stably bind the receptor, when the receptor ceases to be expressed, or when signaling molecules are down-regulated or otherwise inactivated. However, mutations in even one of the molecules involved in regulating these pathways can provide sustained growth signals in the absence of the initiating cytokine. Among many examples, there is a translocation between chromosome 2 and chromosome 5 (t(2;5)) that is present in almost half of human anaplastic lymphomas. The translocation creates a fusion protein between the nucleophosmin gene (NPM1) and the anaplastic lymphoma kinase gene (ALK), which aberrantly activates the Jak2/STAT5 signaling pathway³⁴ that is normally responsive to various interleukins (IL) including IL-2, IL-3, and IL-6. The genes that encode the normal growth promoting proteins (such as ALK, Jak2, and STAT5) are called proto-oncogenes; the mutated versions that allow cells to gain self-sufficiency from the environmental signals are called *oncogenes*. It is important to note that not all growth-promoting genes have the capacity to become oncogenes and that the outcome of oncogenic activation is most commonly apoptosis, unless there are additional events that promote stable transformation and survival.

Insensitivity to Anti-Growth Signals

To maintain homeostasis, cells also must integrate anti-growth signals from the environment. As noted in the previous section, quiescence in non-hematopoietic cells is enforced by signals delivered by contact inhibition. Hematopoietic cells, on the other hand, utilize cell-cell contacts to maintain interactions within the niche and

to regulate the timing and intensity of hematopoiesis, inflammation, and immunity.²⁴ Stop signals are usually delivered and integrated by the products of tumor suppressor genes, which derive their name largely from the observation that their inactivation facilitates tumor formation. Tumor suppressor genes balance the activity of growth-promoting proto-oncogenes and tend to act in tandem with these in most biochemical pathways. Loss of function of one or more tumor suppressor genes occurs in virtually every cancer, with inactivation of p53, RB-1, PTEN, or CDKN2A each seen in >50% of all tumors. Each of these pathways may contribute to the pathogenesis of bone marrow-derived tumors in companion animals, and their dysfunction also may be predictive for outcomes.^{5,15,16,23}

Evasion of Apoptosis

Apoptosis, or programmed cell death, is the imprinted outcome for every cell in multicellular organisms. Survival requires support from extrinsic (environmental) factors, as well as precise balance of cellular energetics and metabolism. Bone marrow derived cells normally undergo apoptosis when concentrations of survival factors (stem cell factor, IL-3, IL-7, etc.) or nutrients are limiting, or when there are severe disruptions to cellular bioenergetics. Evasion of apoptosis is an essential acquired feature of all cancers, and it can result from loss of pro-apoptotic tumor suppressor genes such as *p53* or *PTEN*, or by gain of function of anti-apoptotic genes such as *BCL2*.

Limitless Replicative Potential

Immortalization is another essential feature of cancer. The genetic program limits the number of times a cell is able to replicate, the so-called Hayflick limit, that when reached induces replicative senescence. Induction of replicative senescence does not induce death; cells maintain energetic homeostasis and remain functional, but they undergo significant genetic changes characterized by telomere erosion. Cells that are able to replicate must maintain the integrity of telomeres, “caps” made of repetitive DNA sequence that protect chromosomes from destruction. Solid tumors acquire immortalization predominantly by activation of the telomerase enzyme system and the consequent maintenance of telomere integrity. In hematopoietic cells, telomerase activity seems to be retained longer than in other somatic cells, so it is possible this facilitates immortalization in lymphoma and leukemia.²⁸

Sustained Angiogenesis

The process of angiogenesis requires the coordinated action of a variety of growth factors and cell-adhesion molecules in endothelial and stromal cells. So far, vascular endothelial growth factor-A (VEGF) and its receptors comprise the best-characterized signaling pathway in tumor angiogenesis.⁷ VEGF binds two

receptor tyrosine kinases, VEGFR-1 (Flt-1) and VEGFR-2 (KDR, Flk-1). VEGFR-2 is the major mediator of the angiogenic effects of VEGF. However, VEGFR-1 is expressed by some tumor cells and may mediate chemotactic signals, thus potentially having a role in cancer growth. The expression of VEGF is up-regulated by hypoxia and inflammation. The transcription factor hypoxia inducible factor-1 (HIF-1), which is part of a pathway that also includes regulation by the von Hippel-Lindau (VHL) tumor suppressor gene, is a major regulator of VEGF expression. Under conditions of normal oxygen tension, VHL protein targets HIF for degradation; under low oxygen conditions, HIF increases and VHL-mediated degradation is reduced, permitting upregulation of VEGF. Other signaling molecules that contribute to angiogenesis, include platelet-derived growth factor- β (PDGF- β) and its receptor (PDGFR), and the angiopoietins. PDGF- β is required for recruitment of pericytes and maturation of new capillaries. Recent studies also document the importance of tumor-derived PDGF in recruitment of stroma that produces VEGF and other angiogenic factors (Fig. 61.2).

Angiogenesis appears to be important in hematopoietic tumors. Patients with chronic lymphocytic leukemia responded to treatment with anti-angiogenic compounds,²⁵ followed by similar success for some patients with multiple myeloma.¹¹ Furthermore, different histologic types of human non-Hodgkin lymphoma show different patterns of angiogenesis, and these can

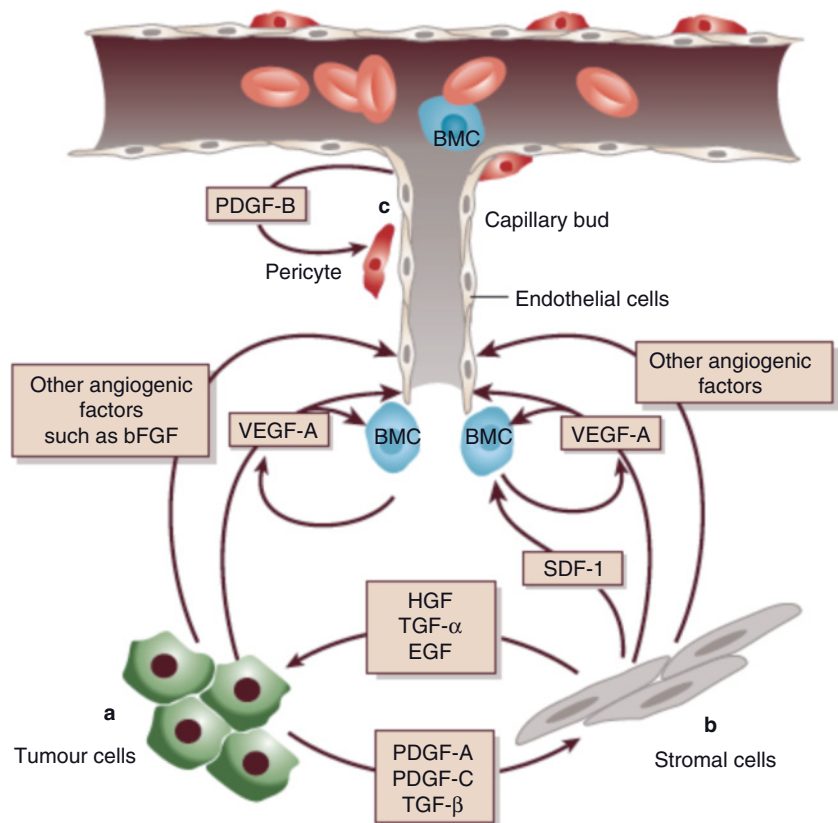
predict outcomes. One study has shown that microvessel density is similarly correlated with aggressive behavior of canine lymphoma,³² and similar findings have been reported for other blood-derived and solid tumors of dogs such as mast cell cancer and mammary cancer.^{31,33}

Invasion and Metastasis

The role of genetic events in invasion and metastasis is still incompletely understood. The classical model of metastasis proposed by Fidler⁸ suggests a stepwise acquisition of assets that enables cells to leave the primary tumor site, travel through blood or lymph, invade stroma in favorable locations, and thus become re-established at distant sites. More recent work suggests that most tumors possess the ability to dislodge cells that travel to distant sites, and the ability of such cells to survive in capillary beds may be the most important step in the metastatic process.¹²

Bone marrow-derived cells have intrinsic properties that allow them to travel throughout the body, traffic through all major organs, and home to areas of inflammation. Thus, bone marrow-derived tumors are inherently metastatic. Nevertheless, hematopoietic tumors that are cytologically indistinguishable can have distinct and preferential tissue distribution. We do not fully understand what events make leukemic cells stay in the circulation, while cells from corresponding

FIGURE 61.2 Molecular and cellular components of the tumor microvascular environment. (a) Tumor cells produce VEGF and other angiogenic factors such as basic fibroblast growth factor (bFGF), angiopoietins, and interleukin-8. These stimulate resident endothelial cells to proliferate and migrate. (b) An additional source of angiogenic factors is the stroma. This is a heterogeneous compartment, comprising fibroblastic, inflammatory and immune cells. Recent studies indicate that VEGF, as well as chemokines produced by tumor-associated fibroblasts, recruit bone-marrow-derived angiogenic cells (BMC). A well-established function of tumor-associated fibroblasts is the production of growth/survival factor for tumor cells such as epidermal growth factor (EGF), transforming growth factor- α (TGF- α), hepatocyte growth factor (HGF), and heregulin. (c) Endothelial cells produce PDGF-B, which promotes recruitment of pericytes in the microvasculature after activation of PDGFR. Tumor cell death will lead to dissolution of blood vessels and release of endothelial cells into the circulation. (Reproduced from Ferrara N, Kerbel RS. Angiogenesis as a therapeutic target. *Nature* 2005;438:967–974, with permission. ©Macmillan.)



lymphomas (or myeloid sarcomas) with virtually identical molecular signatures stay confined to lymphoid or visceral organs.

MOLECULAR EVENTS IN CARCINOGENESIS

Various models have been proposed to explain how cells acquire the features that lead to neoplastic transformation and eventually to clinical cancer. One model describes the process as stepwise accumulation of mutations that reduce constraints on growth and eventually promote transformation. Although this model is overly simplistic and technically flawed it is nevertheless useful to convey the events that lead to carcinogenesis.

Initiation, Promotion, Progression

Unlike diseases due to single gene defects, cancer is a complex, multigenic disease. The initiation, promotion, and progression model was among the first to propose a sequential progression of mutations that could account for cancer.³⁹ In this model, a genetic event would endow a somatic cell with limitless replicative potential or another growth or survival advantage from other cells in its environment (initiation). Alone, this would not be sufficient to give rise to a tumor, as the cell would remain constrained by environmental factors. A second event would further add to the cell's ability to out compete its neighbors in this environment, leading to its potential expansion into a recognizable tumor mass (promotion). Finally, a third event would reinforce the cell's malignant potential (invasion, tissue destruction and metastasis), leading to clinical disease (progression). It is important to note that an "event" is not equivalent to a single mutation, but rather is more likely to represent a series of mutations that act in concert to alter the cell's functional and morphological phenotype.

Genetic Instability

The stepwise model of clonal evolution is satisfying because it can be correlated with discrete pathological changes in tumor progression, especially for epithelial tumors where such progression can be seen in lesions that go through stages of hyperplasia, atypical hyperplasia (dysplasia), adenoma, carcinoma in situ, invasive carcinoma, and metastatic carcinoma. However, analysis of tumor genomes even in early stages usually shows aneuploidy (abnormal DNA copy number) as well as chaotic changes indicative of multiple numerical and structural DNA abnormalities. Similar abnormalities first noticed by Boveri more than 100 years ago in studies of sea urchin cells, which are described in a recently reprinted, annotated version of the original 1914 article translated into English,³ led him to formulate the aneuploidy theory of cancer. We know now that aneuploidy is especially evident in solid tumors;

based on this, Loeb proposed the existence of a "mutator phenotype" where cells are predisposed to undergo multiple mutations, some of which inevitably lead to cancer.²⁰ Some tenets of his hypothesis appear to be correct; however, direct measurements of mutation rates of sporadic tumors are much lower than those predicted if a "mutator phenotype" was operative in these tumors.

Still, genetic instability is a hallmark of most tumors, and while it can be partly explained by increased errors in DNA replication and chromosomal segregation in cells that are rapidly dividing, other mechanisms are clearly operative, involving telomeres and telomerase, as well as mechanisms of DNA replication and repair and chromosomal segregation. Although many of these changes are not recurrent and appear to be random products of instability, some may in fact contribute to proliferative crisis.²¹ It is possible that the initiation events for many tumors occur early in life during highly proliferative stages of tissue growth and remodeling but they become evident late in life when a last series of mutations allows the transformed cell to reach this crisis stage. As we inferred above, hematopoietic tumors seem to avoid the chaotic chromosomal instability associated with solid tumors. We do not fully understand the reasons for this, although it may be partly due to intrinsic protective mechanisms associated with the proliferative rate of bone marrow precursor cells.

Epigenetic Events

Another observation is that events leading to cancer need not necessarily be caused by mutational events, but instead can be caused by epigenetic changes. Epigenetic events are those that can alter phenotype without changing the genotype. Two well-characterized epigenetic mechanisms regulate gene expression. Gene silencing can occur by methylation of CpG residues in promoter regions, as well as by histone deacetylation. Both of these events interfere with the transcriptional machinery and repress gene expression. Silencing specific genes by methylation is implicated in numerous cancers of humans and animals.³⁰ One important observation is that most (or all) genes that are subject to silencing by methylation in specific cancers (e.g. *CDKN2A* in T cell leukemia) are commonly inactivated by mutation or deletion in other cancers (e.g. *CDKN2A* in melanoma).

Genomic imprinting presents a unique example where heritable epigenetic changes influence cancer predisposition. Genomic imprinting refers to a pattern of gene expression that is determined by the parental origin of the gene; in other words, unlike most genes where both parental alleles are expressed, only one allele of an imprinted gene is expressed and the other one is permanently repressed. Epigenetic changes in Wilms' tumor and in heritable colon cancer (among others) alter the expression of the imprinted allele, leading to loss of imprinting that causes overexpression of the insulin growth factor-2 (*IGF2*) gene.³⁰

CANCER STEM CELLS

Unlike diseases that arise from single gene defects, cancer is a complex, multigenic disease. In fact, a sequential progression of mutations is required to produce a tumor cell. We can think of many genes contributing to, rather than causing, the origin and progression of cancer. This model is consistent with many aspects of the natural behavior of tumors, and to some extent, it can predict risk and outcome.

A competing theory now exists whose main tenet is that self-renewal is limited to a small population of cancer stem cells.^{2,4,10} The existence of cancer stem cells is documented; they are characterized both by peculiar phenotypes and by defined sets of mutations of a small number of genes.¹⁰ Other mutations then endow their progeny with limited or extensive capacity to undergo programmed differentiation, hence resulting in distinct clinical phenotypes such as acute and chronic leukemias or high-grade and low-grade solid tumors. The strongest evidence for cancer stem cells comes from hematopoietic tumors, but cancer stem cells also have been identified in various solid tumors.³⁵ The origin of cancer stem cells is among the most important contemporary topics of cancer investigation. Cancer stem cells may arise from mutations that occur in bona fide stem cells, they may arise by de-differentiation of somatic cells that acquire mutations endowing them with stem cell-like properties, or they may develop by fusion of a transformed cell and a bone marrow-derived stem cell. In companion animals, putative cancer stem cells have been identified in hemangiosarcoma,¹⁴ osteosarcoma,⁴⁰ brain tumors,³⁶ and possibly lymphoma.

ADAPTIVE EVOLUTION IN THE TUMOR MICROENVIRONMENT

The clonal evolution theory²⁷ addresses the significance of sequential genetic changes providing growth and survival advantages, but to this we must add the fact that, in addition to these self-sufficient events that influence growth and survival, tumor cells must also evade predators (e.g. inflammation and the immune system). Tumors have the capacity to modulate stromal cells to support their own growth by providing a suitable matrix and an abundance of nutrients, while maintaining antitumor responses at bay. Tumors that outgrow the capability of their immediate surroundings to support their growth will seek to become established in other favorable locations. Thus, adaptive evolution likely contributes to the anatomic distribution of hematopoietic tumors much as it does to the metastatic spread of solid tumors.

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Transforming Retroviruses

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- Murine Leukemia Viruses
- Reticuloendotheliosis Virus
- Simian T Cell Lymphotropic Virus
- Walleye Dermal Sarcoma Virus

Acronyms and Abbreviations

ALSV, avian leukosis and sarcoma viruses; ALV, avian leukosis virus; ASV, avian sarcoma virus; ATL, adult T cell leukemia; BLV, bovine leukemia virus; CAEV, caprine arthritis encephalitis virus; CIS, common insertion or integration site; DNA, deoxyribonucleic acid; EIAV, equine infectious anemia virus; ENTV, enzootic nasal tumor virus; FeLV, feline leukemia virus; FIV, feline immunodeficiency virus; GALV, gibbon ape leukemia virus; HAM/TSP, HTLV-associated myelopathy/tropical spastic paraparesis; HIV, human immunodeficiency virus; HTLV, human T-lymphotropic virus; IL, interleukin; JSRV, Jaagsiekte sheep retrovirus; LRT, long terminal repeats; MMTV, mouse mammary tumor virus; MPMV, Mason-Pfizer monkey virus; MuLV, murine leukemia virus; RSV, Rous sarcoma virus; Sag, superantigen; SIV, simian immunodeficiency virus; STLV, simian T cell lymphotropic virus; WDSV, Walleye dermal sarcoma virus.

Oncogenic retroviruses were first identified near the beginning of the 20th century when Rous and colleagues discovered that certain cancers could be transmitted between animals by the inoculation of cell-free extracts. Studies of these agents have resulted in many critical breakthroughs in the field of oncogenesis.

RETROVIRIDAE

Retroviruses are enveloped RNA viruses that replicate via a DNA intermediate that becomes integrated into the host genome. Seven genera of retroviruses are currently recognized.⁵ Retroviruses exist both as exogenous (non-integrated) and endogenous forms (integrated

provirus). Endogenous retroviruses integrated into the host DNA comprise 5–8% of the human genome, and perhaps more than 10% of the genome in other mammals.

General Retroviral Structure

Retroviruses are single stranded, linear, positive sense RNA viruses with 7–13 kb genomes.^{2,5} All retroviruses contain *gag*, *env*, *pol*, and *pro* genes while complex retroviruses also encode a variety of regulatory proteins. Viral RNA is characterized by the presence of R (repeated) and U (unique) regions at both ends of the genome that, once transcribed, result in identical copies being placed at each end of the newly produced DNA in a U3-R-U5 sequence to create long terminal repeats (LTRs). Long terminal repeats are used for integration into the host genome and serve as regulatory sequences for virus production.

Virions are approximately 100 nm in diameter. At the center is a highly condensed form of the genomic RNA in association with the nucleocapsid protein. The virion core also contains viral enzymes such as reverse transcriptase, integrase, and protease. The core is contained within a structural icosahedral capsid and then a matrix shell, both encoded by *gag*. The virion is enveloped by a lipid bilayer, encoded by *env*, that also produces distinctive glycoprotein cell surface spikes that are critical for virus binding and entry.

Retroviral Life Cycle

The retroviral life cycle is complex and includes variations between different retroviruses; however, the basic template is similar.² Virus binds via a specific cell-surface receptor. Retroviral receptors are diverse and include ion transporters, cell differentiation markers, chemokine receptors, the transferrin receptor, and Fas-like receptors. Internalization and uncoating occurs through fusion of the viral and plasma membrane or via endocytosis.

Reverse transcription of the RNA genome into double stranded DNA uniquely defines the retroviridae. Reverse transcription is a complex, multi-step process initiated by the creation of a negative sense DNA strand. Following degradation of the viral RNA by RNase H, the negative strand serves as a template to create a double stranded linear DNA. The double stranded linear DNA is then transported to the cell nucleus and integrated into the host DNA as a provirus.

Viral RNA and protein are expressed from the integrated provirus using the normal cellular processes of transcription and translation. Transcription by the RNA polymerase II system results in a complete unspliced viral genomic RNA that can serve as the genetic material to be packaged into progeny virions or as the template for production of viral proteins. The latter may require RNA splicing and processing of precursor proteins.

RNA packaging into developing virions is driven through Gag interactions. Gag also controls the assembly of the viral protein products whether it occurs at the plasma membrane or in the cytoplasm. Virions assembled in the cytoplasm are then transported to the membrane for envelopment and release. During the process of release, host membrane proteins are frequently incorporated into the virion envelope and several have been shown to be functionally significant.

CLASSIFICATION OF THE FAMILY RETROVIRIDAE

The current classification by the International Committee on Taxonomy of Viruses uses physical and morphologic properties of the virion, organization and replication of the genome, and biologic properties such as host range to classify viruses.⁵ The family Retroviridae contains two subfamilies. The Orthoretroviruses contain six of the seven genera while Spumaretroviruses comprise only the spumaviruses.

Alpharetroviruses

Alpharetroviruses are simple retroviruses that encode only *gag*, *pro*, *pol*, and *env*. The *pro* and *gag* genes utilize the same reading frame. These C-type virions are assembled at the plasma membrane and do not appear to produce intermediate stage cytoplasmic particles. The virion has a centrally-located, condensed, spherical core and surface projections of intermediate prominence. Key examples of this genera include avian leukosis virus (ALV), Rous sarcoma virus (RSV), and avian sarcoma virus (ASV).

Betaretroviruses

Betaretroviruses are also simple retroviruses but have a B- or D-type virus morphology; they are assembled within the cytoplasm, transported to the plasma membrane, and become enveloped during budding. B-type virions are characterized by a condensed, eccentric core and have prominent surface envelope projections. The *gag*, *pro*, *pol*, and *env* genes are found in different reading frames. Viruses of significance include mouse mammary tumor virus (MMTV), Mason-Pfizer monkey virus (MPMV), Jaagsiekte sheep retrovirus (JSRV), and enzootic nasal tumor virus of sheep (ENTV).

Gammaretroviruses

Gammaretroviruses are also simple C-type retroviruses; however, the *pol*, *gag* and *pro*, sequences share a reading frame. Gammaretroviruses comprise the largest number of species and infect mammalian, avian, and reptilian hosts. This genus also contains a number of replication defective endogenous viruses. Key gammaretroviruses include feline leukemia virus (FeLV), the murine leukemia viruses (MuLV), and the avian reticuloendotheliosis virus group.

Deltaretroviruses

Deltaretroviruses are complex C-type retroviruses that contain *tax* and *rex* regulatory genes in addition to *gag*, *pro*, *pol*, and *env*. Protein expression requires frame shifts over several different reading frames. Bovine leukemia virus (BLV) and human T-lymphotropic virus (HTLV) are the best-known examples.

Epsilonretroviruses

Epsilonretroviruses are complex C-type retroviruses that contain one or more additional open reading frames. The prototype virus is Walleye dermal sarcoma virus (WDSV). Two other Walleye viruses are included in the genus and two additional fish viruses are proposed for inclusion.

Lentiviruses

Lentiviruses are complex retroviruses that are further categorized into five groups based on the host species they infect: primates, cats (domestic and non-domestic), horses, cattle, and sheep/goats. Virions have a distinctive conical morphology. Genomes contain a variable number of accessory genes that regulate viral replication. This genus includes several well-studied viruses including human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), equine infectious anemia virus (EIAV), caprine arthritis encephalitis virus (CAEV), and Maedi-Visna virus. Lentiviruses are characterized by the relatively long period between infection and disease development.

Spumaviruses (Foamy Viruses or Syncytial Viruses)

Spumaviruses are complex retroviruses with immature C-like virions that have a less condensed core and more prominent surface spikes. Virions are assembled in the cytoplasm and then bud through the endoplasmic reticulum as well as the plasma membrane. Newly formed virions uniquely contain large amounts of reverse transcribed DNA. While spumaviruses establish persistent infection in a number of species including humans, non-human primates, cattle, horses, and cats, they are not typically associated with clinical disease.

OVERVIEW OF RETROVIRUS-INDUCED DISEASE

Immunodeficiency

Modification of the immune system by viral proteins can enhance carcinogenesis through several mechanisms including disruption of tumor surveillance, atypical stimulation of the immune system, and alteration of the microenvironment (see Chapter 55). The association of lentivirus-induced immunodeficiency and cancer has been well established in several systems such as

HIV infection and the development of Kaposi sarcomas and B cell lymphomas.

Leukomegenesis

One of the most common mechanisms of retroviral-induced leukomegenesis is through insertional activation, which is the activation of proto-oncogenes following proviral integration.

Direct Cytopathicity

Several viruses produce gene products that are directly cytopathic to the host. Examples include the neurologic damage induced by the Env SU subunit of some MuLVs, wasting syndrome induced by many of the avian viruses, and induction of cytopathicity by FeLVs with mutation in the Env SU subunit.

Host Determinants of Pathogenicity

Alterations in host genes can influence the progression of retroviral infection, particularly those that control the target cell availability through cell cycling, tumor suppression genes, and growth factors. Similarly, mutations within the genes responsible for the immune response can alter pathogenicity.

Stimulation of Host Cell Proliferation

While the primary means of inducing host cell proliferation is through insertional activation, some oncogenic retroviruses use other mechanisms. One of the best understood is the superantigen (Sag) protein of MMTV that binds and activates T cells through common (non-antigen specific) areas on MHCII molecules. Alternatively, the spleen focus-forming viruses and the Env helper protein of MuLV activate erythroid proliferations through binding of the erythropoietin receptor.

Oncogenic Transformation

Retroviruses can induce oncogenic transformation through a number of mechanisms covered in detail in the next section.

RETROVIRUS-INDUCED ONCOGENES

Oncogenes are genes whose products contribute to the formation of cancer. Oncogenes arise as mutated forms of normal cellular genes (i.e. proto-oncogenes) coding for key proteins involved in growth control, development, and differentiation.

Role in Cancer

Carcinogenesis is a multistage process. Oncogenes may have direct or indirect roles at several stages in this process. Direct transformation occurs when a viral gene product interacts with a cellular gene or gene product

resulting in loss of internal regulation of normal proliferation, cell cycle, or cell death and neoplastic transformation in that cell. Indirect transformation occurs when the virus alters cells other than the tumor cell resulting in loss of external regulation. The oncogene may transform only a specific cell type or it may have more global effects.

Non-Acute

Non-acute transforming retroviruses are replication competent viruses that induce neoplasia after a latent period. These viruses do not typically induce morphologic transformation in culture. Oncogenesis is often initiated as proto-oncogenes are activated after viral integration. There are two general mechanisms by which this occurs. In promoter insertion, viral transcription “reads through” to the proto-oncogene each time the virus is replicated resulting in over-expression of the host gene. The second mechanism is enhancer activation in which the proto-oncogene promoter is activated by a viral enhancer.

Acute

Acute transforming retroviruses derive from non-acute retroviruses but have acquired an altered host proto-oncogene. Because they carry oncogenes, these viruses induce rapid transformation and tumor growth. Most acute transforming retroviruses are replication-defective and require replication-competent viruses for propagation. These host-derived oncogenes include growth factors, tyrosine and serine/threonine kinases, transcription proteins, and G-protein signaling proteins.

Mechanisms of Action

The primary mechanisms of oncogenic transformation are through transduction, cis-activation, and trans-activation. Less commonly, viral proteins such as Env may directly stimulate cell proliferation, interfere with cell cycle checkpoints, or inhibit surveillance for tumor cells.

Transduction

During replication, retroviruses can acquire host sequences through recombination errors. These viruses are usually defective and require helper viruses for replication. The transforming power of recombinant viruses depends on the cellular gene they have acquired, but many viruses with rapid transformative ability utilize this mechanism.¹¹ In general, transduction is uncommon in natural infections but is a useful research tool for studying oncogenesis in the laboratory.

Cis-Activation

Cis-activation occurs when a provirus inserts near a proto-oncogene. Activation of the proto-oncogene

occurs by promoters or enhancer elements in the viral LTR. Cis-activation is commonly used by simple retroviruses.

Trans-Activation

Trans-activation occurs as a virally encoded protein such as Tax interacts with host transcriptional factors, thereby altering expression of normal cellular genes.

PROTOTYPICAL AND WELL-CHARACTERIZED ONCORETROVIRUSES

Avian Leukosis and Sarcoma Viruses

The avian leukosis and sarcoma viruses (ALSVs) cause tumors in a variety of avian species. ALVs are simple retroviruses that are transmitted both horizontally and vertically. However, while a large percentage of the flock may be infected, neoplasia develops in only a small percentage. Vaccination strategies have not been successful, making eradication of ALV from the breeding stock a critical control step. In the early 1980s, eradication of the five primary envelope subgroups had been reasonably successful. However in the mid 1980s, a highly pathogenic myeloid tumor-inducing subgroup J virus emerged to become one of the major infectious diseases facing the poultry industry.

Exogenous ALVs are transmitted as infectious virions while endogenous ALVs are incorporated into the host genome and transmitted genetically. The number of endogenous viruses varies among avian species and the biological significance of endogenous viruses is not entirely clear. Some forms appear to protect the host from infection by newly acquired ALV infection through receptor interference. However, endogenous viruses may also have a negative effect on the host.

ALVs are well-described oncogenic viruses. Both non-acute and acute transforming forms are recognized. Non-acute transforming viruses induce tumors that appear late in the period of infection. Typically, oncogenesis is induced through the insertion of the ALV genome near a proto-oncogene resulting in cis-activation. Lymphoid leukosis is one of the best-described examples. In lymphoid leukosis, the B cell *c-myc* gene is activated resulting in a multistage lymphomagenesis that produces neoplastic lymphoblasts resistant to apoptosis. Because multiple follicles can be affected nearly simultaneously, the disease can appear to be polyclonal.

In contrast, acute transforming ALVs contain one or more viral oncogenes. These ALVs tend to be replication incompetent due to mutations (usually deletions) within the structural genes and require co-infection with a non-defective helper virus. Several ALV strains carry the *myc* oncogene that tends to induce myelomonocytic neoplasia. Avian sarcoma viruses also tend to be acutely transforming. The best characterized is Rous sarcoma virus which encodes a protein kinase through *src* viral oncogene.

In addition to their known neoplastic induction, certain ALVs serve as a model of viral associated obesity. For example, the Rous-associated virus type 7 ALV induces a syndrome of hypertriglyceridemia and hypercholesterolemia.

Bovine Leukemia Virus

Bovine leukemia virus (BLV) is a type C deltaretrovirus that infects cattle and sheep. Bovine leukemia virus replication is primarily dependent on cell proliferation and mitotic division. Virus infection stimulates a polyclonal expansion of B cells, called persistent lymphocytosis, in approximately one-third of infected animals. In cattle, only a small percentage of infected animals develop bovine enzootic leukosis, a polyclonal, but neoplastic, B cell expansion. A higher rate of neoplasia is seen in experimentally infected sheep. Transmission is primarily horizontal through the transfer of infected cells by direct contact and possibly through blood-sucking insects. Vertical transmission is less common but may occur in utero, at parturition, or through milk ingestion.

Bovine leukemia virus is related to the primate lymphotropic viruses but has over 40% phylogenetic divergence. While the primate viruses infect T cells, BLV primarily infects B cells, particularly those expressing surface IgM, but also can infect monocytes and macrophages. Bovine leukemia virus encodes both Rex and Tax proteins, Tax being the primary oncogenic protein. Transmission by cell-free virus is inefficient and cell-to-cell transmission by cell fusion predominates. After infection of a new cell, BLV integrates randomly into the host genome. Virus replication is primarily through the production of daughter cells via mitotic division.

Feline Leukemia Virus

Feline leukemia virus (FeLV) is a simple retrovirus that infects domestic cats as well as non-domestic felids. FeLV has high sequence homology with MuLV, supporting the hypothesis that infection of cats resulted from MuLV cross-species transmission. Three genotypes of FeLV have been described: types A, B, and C. Type A FeLV is the dominant virus in nature. This exogenously transmitted retrovirus is ubiquitous, readily transmitted, and highly conserved. Types B and C result from mutation and recombination of type A FeLV with endogenous retroviral sequences. The variability in viral recombination corresponds with variability in the pathogenesis and disease outcomes including aplastic anemia, T cell lymphoma/leukemia, myeloid leukemia, and immunosuppression.

Feline leukemia virus is transmitted horizontally, primarily through saliva, biting, and other close contact. Vertical transmission occurs both in utero and by ingestion of infected milk. Susceptibility is inversely correlated with age; thus the highest potential for transmission and infection occurs during the first 4–6 months of age. Approximately 30% of infected cats develop persistent

viremia and secondary diseases including immunodeficiency, hematopoietic and other neoplasia, and opportunistic infections. The majority (60–65%) of all infected cats rapidly or eventually clear the virus after infection due to a robust immune response, minimal virus exposure, or a combination of the two, and remain antigen negative by enzyme-linked immunosorbent assay (ELISA). A small percentage of cats develop a transient antigen-positive viremia but then become persistently aviremic and antigen negative. Finally, 5–10% of all infected cats may develop an “atypical” infection due to localized or sequestered infection and have mixed or inconsistent test results. These cats may maintain a life-long latent infection, but may progress to outright viremia or viral clearance. Latent FeLV may be reactivated through secondary diseases or treatments that result in impairment of the immune system.

Gibbon Ape Leukemia Virus

Gibbon ape leukemia virus (GALV) is genetically related to simian sarcoma associated virus, murine leukemia virus, and feline leukemia virus. Gibbon ape leukemia virus is a highly oncogenic C-type retrovirus that induces myeloproliferative disorders, particularly in juvenile animals. To date, four strains of GALV have been identified from captive gibbons. Because the virus has not been identified in wild animals, it is uncertain if GALV is of simian origin or represents an endogenous retrovirus transferred between species. Because GALV infects a wide variety of cell types from different species, several GALV-based retroviral vectors have been developed for gene transfer.

Human T Cell Lymphotropic Viruses

Type 1 and type 2 human T cell lymphotropic virus (HTLV) are closely related viruses that share significant nucleotide similarity but also have important differences. Recently, additional members of the HTLV family, HTLV-3 and HTLV-4 have been identified.⁶ The HTLVs are complex retroviruses with several regulatory and accessory genes. The HTLVs are genetically stable viruses whose replication is limited by the immune response. HTLV replication primarily occurs through clonal replication of infected cells. HTLV-1 is endemic in South-East Asia including Japan, the Caribbean, Melanesia, as well as parts of South America, central Africa, and the Middle East. HTLV-1 infection can result in adult T cell leukemia (ATL) and HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP); however, only a small percentage of infected individuals develop clinical disease.

Jaagsiekte Sheep Retrovirus and Enzootic Nasal Tumor Virus

Jaagsiekte sheep retrovirus (JSRV) is the causative agent of pulmonary adenomatosis in sheep. Pulmonary adenomatosis serves as a transmissible model of human

bronchioalveolar carcinoma. It is a simple retrovirus but includes an additional gene, *orf-x*. The mechanism of JSRV induced oncogenesis is not well defined but both *orf-x* and *env* have been proposed to play a role. The virus spreads horizontally through infection of type II pneumocytes and Clara cells in the lower respiratory tract. The time to tumorigenesis ranges from months to years, but disease progresses rapidly once clinical signs develop.

Enzootic nasal tumor virus (ENTV) has >95% genetic similarity with JSRV. While JSRV induces tumors in the lower respiratory tract, ENTV causes tumors in the nasal cavity.

Mouse Mammary Tumor Virus

Mouse mammary tumor virus (MMTV) induces mammary adenocarcinomas and, less commonly, T cell lymphoma. MMTV is transmitted genetically through the germ line and via infected breast milk. MMTV has been classified as a simple betaretrovirus but encodes Rev, a self-regulatory mRNA export protein related to the Rev protein of HIV.^{3,7} MMTV also encodes *sag*, a superantigen that uniquely stimulates viral replication. B cells in the gut are the initial target for viral infection. B cell expression of the superantigen activates bystander CD4+ T cells that, in turn, induce clonal expansion of *Sag* expressing B cells responsible for trafficking to the mammary gland and infecting mammary epithelial cells.

Mouse mammary tumor virus tumorigenesis occurs primarily through viral insertion near a proto-oncogene and subsequent activation. Endogenous viruses are frequently truncated or defective; however, those mice with a functional *sag* gene have some degree of protection against infection with exogenous MMTV because the T cells that would normally react against the superantigen have been deleted during thymic maturation.

Recently, several sequences have been detected within the human genome and human cancers that have very high sequence homology with MMTV. Although somewhat controversial, this has led to speculation that these elements may play a role in human breast cancer and lymphoma as well as other diseases such as primary biliary cirrhosis.^{1,4,8,9,10,12} While additional work is necessary to establish a causative link between MMTV-like sequences and these diseases, current studies are shifting our understanding of the role of endogenous retroviruses and retroelements.

Murine Leukemia Viruses

There are several strains of well-characterized murine leukemia viruses (MuLVs) including Friend, Moloney, and Rauscher viruses. MuLVs induce a wide spectrum of malignancies including T cell lymphoma/leukemia, myeloid leukemia, and erythroleukemia. These viruses can also affect the nervous system. The tumorigenic effects are driven by the LTR U3 region while neuro-pathicity is mediated by the Env SU. These simple retroviruses are further classified by host range and

replication capacity. Ecotropic viruses replicate only in their natural host. Amphotropic or polytropic viruses replicate in both rodent and non-rodent species. Xenotropic viruses infect only non-murine cells. Wild rodents frequently harbor non-ecotropic viruses. Because these non-ecotropic viruses efficiently infect human cells, they make efficient gene delivery vectors.

Reticuloendotheliosis Virus

Reticuloendotheliosis virus (REV) is a simple gamma-retrovirus that infects a number of avian species. Serologic studies suggest that the virus is prevalent in both chickens and turkeys, and appears to be transmitted vertically and horizontally. The name reticuloendotheliosis virus arises from the acute reticulum cell neoplasia seen in chickens experimentally inoculated with a unique replication defective laboratory strain containing the *rel* oncogene. However, most clinical isolates lack oncogenes and are replication competent. Reticuloendotheliosis virus induces three distinct clinical syndromes including a non-neoplastic runting syndrome associated with bursal and thymic atrophy, a variety of acute neoplastic diseases involving multiple organs, and chronic B and T cell lymphomas.

Simian T cell Lymphotropic Virus

The simian T cell lymphotropic viruses (STLVs) are complex deltaretroviruses closely related to HTLV. The genetic relatedness between STLV-1 and HTLV-1 supports the hypothesis that HTLV-1 originated from cross-species STLV transmission. STLV-1 infects over 20 species of Old World primate species while STLV-2 has a much more limited species range. Clinical disease is uncommon in STLV infected primates and, when seen, tends to occur following chronic infection.

Walleye Dermal Sarcoma Virus

Walleye dermal sarcoma virus (WDSV) is the best-characterized piscine retrovirus and is endemic in walleye throughout North America. It is a complex retrovirus with at least three accessory genes. Walleye dermal sarcoma virus replication and the induced dermal sarcomas have a unique cyclical pattern. Walleye dermal sarcoma virus-induced dermal sarcomas are benign tumors that develop in the fall when virus production is low. In the spring, virus replication increases and these tumors concurrently regress. These findings suggest that virus replication is dependent on host endocrine or immunologic changes associated with spawning and/or changes in water temperature.

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Bone Marrow-Derived Sarcomas

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Tumors arising from Mesenchymal Progenitor Cells
Hemangiosarcomas

Acronyms and Abbreviations

AS, angiosarcoma; HSA, hemangiosarcoma; HSC, hematopoietic stem cell; LSC, leukemia stem cell; MSC, mesenchymal stem cell; OSA, osteosarcoma; OSX, osterix; PPAR- γ , peroxisome proliferator-activated receptor-gamma; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

Traditionally, tumors have been divided into two distinct categories according to their ontogeny. One category considers all solid tumors, which are those that do not originate from hematopoietic progenitors, and the other includes “liquid” tumors, so called because they arise from hematopoietic progenitors and have a tendency to exist in the circulation. Despite the colloquial misnomer (hematopoietic tumors can form solid masses in lymphatic organs, viscera, skin, etc.), this separation is the basis not only for diagnostic algorithms, but even for clinical specialization among medical oncologists and pathologists. While this simplified scheme has clinical relevance in many situations, it may overlook some complexities in the biological relationships between hematopoietic and mesenchymal tissues that are relevant to our understanding of tumor biology.

The shared origin of mesenchymal tissues and blood cells from the embryonic mesoderm has been appreciated for over a century. However, it is only recently that multipotent mesenchymal stem cells (MSCs) have been isolated from bone marrow.²⁶ Most investigators believe that MSCs are distinct from hematopoietic stem cells (HSCs), although this distinction is blurred by the acceptance, at least by some, of specialized progenitor cells that retain the potential to give rise to both mesenchymal cells and hematopoietic elements. For example, two groups showed independently that stem cells, which give rise to chronic myelogenous leukemia and which harbor the prototypical BCR-Abl (Philadelphia chromosome) translocation, can give rise to apparently normal vascular endothelial cells *in vivo* and under specialized culture conditions.^{10,13} These findings

suggest that a common multipotent hemangioblast precedes the formation of lineage committed hematopoietic progenitors and endothelial progenitors. Other experimental evidence is consistent with pre-adipocyte trans-differentiation into a macrophage-like cell,^{5,18} further demonstrating the potential for plasticity of hematopoietic and mesenchymal precursors.

The plasticity of MSCs in the laboratory, as well as unique properties of mesenchymal tumors arising from bone marrow, has forced us to consider additional complexity within the bone marrow progenitor pool. Experimental evidence *in vivo* also supports the existence of multipotent MSCs from bone marrow. They are capable of differentiating along multiple lineages, including chondrocytes, osteoblasts, adipocytes, fibroblasts, and endothelial cells, depending on chemical signals, physical interactions with the environment, and potentially in response to mechanical factors such as hydrostatic pressure and fluid-induced shear.¹⁴ Several key transcription factors appear to regulate osteoblastogenesis and adipogenesis in a reciprocal manner. Osteoblastogenesis is enhanced by Runx2 and osterix (OSX) and adipogenesis is stimulated by peroxisome proliferator-activated receptor- γ (PPAR- γ). PPAR- γ may contribute to age-related bone loss²⁶ or to anemia associated with chronic disease.²⁷ Moreover, there is evidence to support the existence of bone marrow angioblasts or hematoendothelial progenitors that can differentiate into both endothelial cells and hematopoietic elements.

The structure of the bone marrow and the relationship between the stromal and hematopoietic elements are described in Chapter 2.^{22–24,36} Here, we will focus on

the possible significance of shared progenitor cells on non-hematopoietic bone marrow sarcomas.

TUMORS ARISING FROM MESENCHYMAL PROGENITOR CELLS

Various sarcomas may arise primarily from the medullary cavity of bone, potentially due to transformation of a mesenchymal progenitor cell. Osteosarcoma (OSA) is the most common of these tumors in most domestic species, especially the dog. Heterogeneous histologic subtypes (e.g. poorly differentiated, chondroblastic, fibroblastic, telangiectatic, and giant cell type) can present a diagnostic challenge to pathologists and may reflect the diverse paths for differentiation open to a transformed mesenchymal stem cell. It is not clear if the predominant histologic pattern reflects the underlying genetic abnormalities of the transformed cell, or is secondary to environmental influences on the differentiation of neoplastic tissue. Medullary fibrosarcoma, chondrosarcoma, and hemangiosarcoma (see below) are each less common, but are reported with some frequency in dogs, and may in some cases be difficult to differentiate from the more common but phenotypically variable OSA. Although not technically a primary tumor of marrow in veterinary species, myelolipoma is a benign tumor comprising a mix of mature adipose and hematopoietic tissue with occasional bone formation that can be identified in the liver, spleen, and adrenal glands of veterinary species.³⁷ The origin of these tumors is obscure; however, myelolipomas reinforce the interdependence of connective tissue and hematopoiesis.^{8,19}

Hemangiosarcomas

Among the mesenchymal tumors arising from bone marrow, hemangiosarcoma (HSA) may be a special case. Hemangiosarcoma is a unique soft tissue sarcoma that is relatively common in dogs, occurs occasionally in other species, and appears to be ontogenetically related to human angiosarcoma (AS) and Kaposi sarcoma (KS), as all three are presumed to arise from hemangioblastic or endothelial progenitors and share signaling abnormalities.^{7,11,15,34} The highly metastatic behavior and modest response to chemotherapy distinguish these tumors from other soft tissue sarcomas that are locally invasive and generally unresponsive to chemotherapy.

The histologic diagnosis of HSA is usually uncomplicated. It is a tumor of spindle cells that form dilated, tortuous, and leaky capillary spaces or sinusoids that generally contain blood. The abnormal vessel morphology disrupts laminar flow, often leading to clots that can devitalize the growing tumor. This in turn leads to necrosis and eventual rupture and hemorrhage, which are hallmarks of this insidious, progressive disease. It is not unusual for the tumor to be practically lost within large fibrinous blood clots at the time of diagnosis or at post-mortem analysis.

When the diagnosis of HSA is in doubt, it can be readily established by evaluating expression of endothelial cell markers, including CD31, factor VIII-related antigen (FVIII-ra; also called von Willebrand's antigen), and vascular endothelial growth factor receptor-2 (VEGFR-2, also called fetal liver kinase-1 or Flk-1 and kinase domain insert receptor or KDR).^{1,3,11,16,25,35} Recently, our group showed that HSA cells also express the c-Kit receptor (CD117).¹¹ c-Kit is a common marker of early progenitor cells, expressed broadly across various lineages. Surprisingly, we also found that HSA cells express CD34,²⁰ an adhesion molecule that is normally present in hematopoietic progenitors as well as in some endothelial progenitors. The unanticipated finding, which raised questions about the ontogeny of HSA, was the consistent expression of CD45 by HSA cells, since this protein is a reliable marker of hematopoietic commitment.²⁰ Even more perplexing was the presence of various markers associated with myeloid differentiation, such as CD14. This led us to propose that HSA originates from a bone marrow progenitor with hematopoietic potential.

Concurrent work by Yoder et al. documented the existence of an endothelial precursor cell that is an intimate participant in vasculogenesis and angiogenesis, but which originates from a myeloid progenitor.³⁸ These cells are "vasculomimetic", expressing all or most of the proteins normally associated with endothelial ontogeny (e.g. CD31, FVIII-ra, and VEGFR2). In addition, these cells express markers associated with myeloid or hematopoietic lineages, including CD45, CD14, and the colony-stimulating factor-1 (CSF-1) receptor (CSF-1R or CD115), and had strong phagocytic activity against bacteria. Functionally, these cells could migrate along gradients of vascular demand, invade artificial matrices, and create structures that resemble vascular channels, but they did not line blood vessels and could not support microcirculation in a transplanted matrix. Yoder et al. also identified a distinct endothelial precursor cell that did not express the hematopoietic markers CD14, CD45, or CSF-1R, and which was capable of lining endothelial capillaries and supporting microcirculation in Matrigel implants.^{4,17,38} This led them to propose that angiogenesis requires at least three cell types. In their model, two of the cells are those described above, which have distinct origins from hematopoietic (myeloid) and hemangioblastic (endothelial) progenitors, and which respectively form and line the vascular channels. The third cell type, which probably originates from a white fat MSC, differentiates into pericytes and other supporting structures.

It is thus possible that multiple lineages contribute to blood vessel formation, including one originating from a restricted angioblastic progenitor that gives rise to the endothelial lining cells and one originating from a myeloid progenitor that is responsible for creating (but not lining) vascular channels. In this scenario, plasticity of adult hematopoietic and mesenchymal stem cells would be limited, differentiation of myeloid progenitors into endothelial-like cells would have to reflect functional rather than ontogenetic plasticity, and we

should consider the possibility that HSA represents a subtype of myeloid sarcoma. We have data to support this possibility. Specifically, HSA may be unrelated to other vascular tumors such as hemangioblastoma and hemangioendothelioma that are characterized by mutations of the von Hippel-Lindau (VHL) gene.¹² Instead, HSA cells down-regulate expression of PLZF, a gene involved in myeloid cell differentiation,^{29,32} and they show enrichment of gene sets involved in differentiation of promyelocytic leukemia cells, dendritic cells, and mast cells.^{6,30,32,33,39} This is significant in light of the poor response to treatments and it may signal the need to revise the therapeutic approach to this disease as a tumor of hematopoietic origin.

However, the data do not exclude the possibility that HSA might arise from a common hematoendothelial progenitor cell and not from a lineage committed myeloid cell. Genes that regulate cellular metabolism, cell cycle and cell signaling, cell-cell interactions, survival and apoptosis, angiogenesis, transcription, and the immune response are among those dysregulated in HSA cells when compared to non-malignant proliferating endothelial cells.³² Up-regulation of proinflammatory genes in HSA could result from selective pressures to create a favorable microenvironment for growth and survival. For example, among genes overexpressed in HSA, fibronectin-1 is involved in wound healing, blood coagulation, and cancer metastasis, and it also increases matrix metalloproteinase activity, which is involved in tumor cell invasion through the extracellular matrix.^{2,9} This is an interesting correlation, since survival of HSA is exceptionally poor due to its exceedingly high metastatic potential. The possibility that fibronectin-1 promotes the metastatic phenotype has been the subject of intense study in other tumors,²¹ but remains to be examined in HSA.

On the other hand, angiogenic and inflammatory signatures might reflect the shared hematoendothelial ontogeny of HSA rather than selection in the tumor microenvironment. Inflammatory infiltrates are commonly seen in this disease, but rather than reflecting recruitment of tumor-associated macrophages and myeloid cells due to inflammation, the presence of leukocytes may actually be derived from the same population of cancer stem cells that give rise to HSA. Recent data suggest that classical cell markers for endothelial and myeloid origin cells are less tissue specific than historically thought,²⁸ supporting the existence of the putative angioblast. Our data support the existence of rare progenitor cells in HSA that behave like cancer stem cells (see Chapter 3). There also is evidence for cancer stem cells that are capable of differentiating along different developmental paths to give rise to endothelial cells in chronic myelogenous leukemia and Burkitt lymphoma,^{10,13,31} although the possibility of vascular mimicry rather than true vascular differentiation cannot be excluded.

A similar dilemma applies to another poorly understood variant of intramedullary HSA, which frequently is associated with osteoid matrix. This tumor may be an ossifying HSA or a telangiectatic osseous sarcoma, but

the possibility that it is composed of vasculomimetic OSA cells cannot be excluded. The shared ontogeny of HSA and OSA allows us to consider an alternative, which is that these tumors arise from a multipotent MSC that can differentiate along the vascular endothelial and the osteogenic lineages depending on micro-environmental cues.

The eventual resolution of these contemporary questions will help to resolve important controversies in cancer biology, may improve our diagnostic accuracy, and may inform the development of effective strategies to treat these intractable cancers in pets and people.

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Classification of Leukemia and Lymphoma

V.E. TED VALLI and WILLIAM VERNAU

Early Systems of Lymphoma Classification
 The Kiel Classification, The Lukes and Collins
 Classification, The World Health Organization
 Classification
 The International Working Formulation for Clinical
 Usage

The European-American Classification of Lymphoid
 Neoplasms
 World Health Organization Classification of
 Hematologic Malignancies

Acronyms and Abbreviations

FISH, fluorescent in situ hybridization; REAL, revised European-American lymphoma classification system; WHO, World Health Organization.

Hematopathology has the dubious distinction of being one of the most chaotic areas of disease classification. In retrospect, the major reason for this is that cell characteristics, on which classical classification systems depend, differ only very slightly and these changes are only detectible by experienced investigators. Thus, early classification systems, based mainly on morphologic characteristics, continued to evolve because it became apparent that cells with similar morphology had different behaviors. The advantage of current classification systems, that are based on disease characteristics, is that the total entity (i.e. cell type and origin, topography of areas affected, and phenotype and genotype) are all included in the definition of the disease.^{11,12,16,17,20,27,34} Many factors have driven development of disease classifications, including recognition that closely related diseases may have widely variable survival rates and that disease subtypes with differing biology could be tied to definable differences in histology and responses to treatment. The evolution in classification of lymphomas has occurred through heated discussion and survived national and geographic preferences. It is immediately apparent that research progress is greatly hampered by competing systems of classification because it cannot be firmly established that people conducting clinical trials are actually dealing with the same disease entity.

An encyclopedic treatment of the history of lymphoma classification is described by Ferry and Harris.⁸ Virchow named leukemia in 1845 and lymphosarcoma

as a distinct form of lymphoid tumor in 1863. Because of some of the similarities between benign hyperplasia and clonal lymphoid disease, Bilroth suggested the term malignant lymphoma in 1871. These terms have persisted to the present with interesting connotations. Renewed interest in lymphoid neoplasms began in the latter part of the 20th century when it was shown that some types of Hodgkin's lymphoma could be cured with irradiation. In veterinary medicine, many have chosen to continue to use the term lymphosarcoma as an all-encompassing term to describe lymphoid neoplasms. While not incorrect, this approach has the disadvantage of separating understanding of animal lymphoid tumors from the advances made in human hematopathology. The current understanding is that some lymphomas, of both B and T cell types, while clonal, are indolent and slowly progressive with the animals occasionally dying of other causes. In that context, these diseases are more conveniently recognized and identified as "indolent lymphomas," thereby appropriately differentiating them for diagnosticians and therapists, from the aggressive lymphomas that should be identified as lymphosarcoma.

EARLY SYSTEMS OF LYMPHOMA CLASSIFICATION

One of the first classifications of lymphoma was that of the American Registry of Pathology: "Classification of

Lymphatic and Reticular Tumors" in 1934.⁴ This classification system included these divisions; lymphocytic (indicating small cell), lymphosarcoma (indicating medium-sized cell), and reticulum cell (including all large cells). The lymphocytic group was subdivided into acute and chronic leukemic forms as well as solid tumors of diffuse and nodular types that were aleukemic. The lymphosarcomas or medium-sized tumors were divided into those that were aleukemic solid tumors and leukemic forms. The reticulum cell sarcomas included leukemic reticulocytoma but also included the monocytic leukemias and aleukemic forms of the same cell types. Reticulum cell sarcoma was a term retained for only the large cell lymphomas.

The major classification systems following that of the American Registry of Pathology included that of Robb-Smith (1938)³¹ in England, whose system was very complex and was based on the concept that there was a benign and malignant counterpart for each cell type recognized within the concept of the reticuloendothelial system. In 1942, Gall and Mallory at the Massachusetts General Hospital reviewed their 1,618 cases of lymphoid neoplasms and provided a new system of classification that included the following list of tumors: reticulum cell sarcoma, stem cell lymphoma, clasmatocytic lymphoma, lymphoblastic lymphoma, lymphocytic lymphoma, follicular lymphoma, Hodgkin's lymphoma, and Hodgkin's sarcoma.¹⁰ Following Gall and Mallory, Jackson and Parker (1939 and 1944) published their classification that subdivided Hodgkin's disease into paragranuloma, granuloma, and sarcoma that rapidly became the standard in the United States and remained so until the mid 1960s.¹³⁻¹⁵ The next major change was the introduction of the Rappaport classification system (1956³⁰ and 1966²⁹) that brought a new parameter to classification based on pattern or architecture. Therefore, this system separated follicular neoplasms from those with diffuse architecture. Rappaport concluded that regardless of cell type all lymphoid neoplasms including Hodgkin's disease have a better prognosis if accompanied by a nodular or follicular architectural pattern. A disadvantage of the Rappaport system was that the specific entity of aggressive small cell lymphoblastic lymphoma was lost and combined with diffuse poorly differentiated lymphocytic lymphoma. Another disadvantage of the Rappaport system was that Hodgkin's lymphoma lost recognition of the subtype with lymphocyte depletion and predominance of Reed Sternberg cells known as Hodgkin's sarcoma. Although now considered a regressive step in terms of understanding of lymphomas, the Rappaport system became the standard in the United States because it required pattern recognition that separated the indolent follicular lymphomas from the clinically more aggressive diffuse lymphomas. A large study at Stanford University in 1974 confirmed that the Rappaport system actually had "clinical significance", causing the system to become the standard in North America.^{7,18}

In the 1960s, it was shown that Hodgkin's disease could be cured with radiation therapy.³² Lukes and Butler in 1966,²⁴ using material from the Armed Forces

Institute of Pathology, described a new classification of Hodgkin's disease in which the older system of Jackson and Parker was modified by dividing the granuloma category into two subtypes of *nodular sclerosis* and *mixed cellularity*. A clinical conference on Hodgkin's lymphoma held in Rye, New York, reviewed previous systems and adopted a modified version of the Jackson and Parker system. This modified version of the Jackson and Parker system rapidly became a world standard, subsequently known as the Rye classification of Hodgkin's disease.²⁶

THE KIEL CLASSIFICATION, THE LUKES AND COLLINS CLASSIFICATION, THE WORLD HEALTH ORGANIZATION CLASSIFICATION

Karl Lennert of Kiel, Germany, much like Robb-Smith of England, looked for malignant counterparts of benign cells. Lennert realized that many lymphomas consisted of cells that resemble those present in benign germinal centers and could be shown to produce immunoglobulin consistent with germinal center B cells.^{22,23,33} The strategy employed in the Kiel classification was based on a proposed scheme of leukocyte differentiation. Most of the entities defined were of B cell type but several T cell lymphomas were also described. In this scheme the lymphomas were grouped according to cytologic features; low-grade lymphomas consisting of those with more dominance of small cells were named ending in "cytes" and high grade tumors with a predominance of large cells were named with terms ending in "blasts". This classification was updated further in 1992²² and the system became widely used in Europe but was never accepted in the United States.

The Lukes and Collins classification, published in 1974,²⁵ was based on the same observations of cell type as the Kiel classification and included what immunologic data were known at the time. A major drawback with this system was it first required lymphomas to be divided into T and B cell lineages but phenotyping was not generally available at that time. Further, there were no data to indicate whether the distinction of phenotype was an important prognostic factor. The Lukes and Collins classification did not recognize histologic pattern as having any influence and contended that cell type alone could predict clinical outcome. Another major problem with this classification was that a single category based on tumors arising from malignant follicles contained a vast majority of all lymphomas. The Lukes and Collins system never achieved wide use.

After the description of the Kiel, and the Lukes and Collins classifications, three others were proposed including a working classification by Dorfman⁶ and a proposal by Bennett and co-workers¹ that became the British National Lymphoma investigation classification. The third was the World Health Organization (WHO) classification that reflected immunology being applied to the Rappaport classification, with large cell lymphomas recognized to be of lymphoid rather than histiocytic origin. The Dorfman and WHO classifications

failed to achieve widespread use, but the Bennett classification was widely used in the United Kingdom.

THE INTERNATIONAL WORKING FORMULATION FOR CLINICAL USAGE

In the mid 1970s, several international meetings were held to resolve the situation without any consensus resulting. The American National Cancer Institute undertook a large-scale blinded review project based on patients who had been enrolled in clinical trials at cancer treatment centers around the world. Their strategy was to review a large number of cases, for which the outcome was known, using each of the six current lymphoma classifications. The goal was to determine which of these classifications most accurately predicted the outcome and thus clinical behavior of each lymphoma subtype. The major finding of the study was that none of the classification systems were superior in predicting survival and that the intra- and inter-observer reproducibility was poor for all of the proposed classification systems. This study resulted in a “working formulation for clinical usage” to “translate” and identify specific cell types between the different classifications.⁵ Essential characteristics of the Working Formulation²⁷ were that it used the Rappaport categories but inserted terminology, primarily from the Lukes and Collins classification, that was accepted to be biologically more correct (i.e. small lymphocytic, rather than well-differentiated lymphocytic; small cleaved cell rather than poorly differentiated lymphocytic; large cell lymphoma rather than histiocytic). A number of inconsistencies remained, including the category of diffuse mixed small and large cell that included follicular center lymphomas that had progressed to the point of becoming diffuse. Large cell lymphomas were divided into immunoblastic types consisting of cells with a single central nucleolus, that were felt to be high grade tumors, and centroblastic types that consisted of cells with peripheralized nucleoli and that were felt to be of intermediate grade, without phenotype being known for either. Novel features of the Working Formulation classification were the division of lymphomas into low-, intermediate-, and high-grade types and with each subtype having a letter designation alphabetically, a-j, meant to simplify terminology. An important inconsistency arising from the proposal was that the clinical grades of the Working Formulation were based on survival, while the histologic grades of the Kiel classification were based on morphologic features of the tumors such as the cell size, nuclear chromatin features, and mitotic rate.

Oncologists tended to treat the entities within the low-, intermediate- and high-grade areas with the same protocols, assuming they were all of similar biology based on survival. One problem with the application of the Working Formulation was that pathologists could not reliably distinguish between immunoblastic cell types, considered to be high-grade, and other large-cleaved or non-cleaved cell types, with what is now called centroblastic nuclear morphology. Despite these

drawbacks, the Working Formulation became widely used in North America. The result was that well over half of all lymphomas could be diagnosed and adequately treated according to the Working Formulation criteria. The lack of specificity grew out of mixing criteria in oncology trials.

By the 1980s and 1990s many new disease entities had been described that were not included in either the Kiel classification system or the Working Formulation. A continuing dislocation was the fact that the Kiel classification was primarily used in European countries while the Working Formulation was primarily used in North America. As a result, many hematopathologists began to design new classifications.²³

THE EUROPEAN-AMERICAN CLASSIFICATION OF LYMPHOID NEOPLASMS

An informal group of 19 hematopathologists from Europe, the United States and Asia had been meeting since the beginning of the 1990s and reached consensus on a new approach to lymphoma classification. It was decided that all available information from each case would be used to define lymphomas. The data included cell morphology, immunophenotype, genetic and clinical features, tumor topography, and clinical behavior of the tumor.⁵ Different criteria were given variable importance in defining each of the lymphoma subtypes. The cellular morphology was always important, and in some cases was the defining feature. Pattern or architecture was singularly important in defining follicular lymphomas. Vascular proliferation was noted to be a key feature in what was called angioimmunoblastic T cell lymphoma and the connective tissue component was of major importance in the nodular sclerosis type of Hodgkin's lymphoma. Some diseases were largely defined by immunophenotype and pattern, such as (B cell) mantle cell lymphoma and marginal zone lymphoma (see Chapter 69). In anaplastic large cell lymphoma, the abundant cytoplasm, remarkable anisokaryosis, and peculiar horse shoe-shaped nuclei were recognized to be of primary diagnostic importance. In some lymphomas, and particularly in primary leukemias, the genetic changes (e.g. chromosomal translocations) were recognized as important. Finally, in some instances, topography was of particular importance.

The emphasis on defining diseases based on utilization of all available information represented a new paradigm in recognizing lymphoma subtypes. A further aspect of this approach, of particular importance to animals, is that poorly described diseases can be given a conditional classification that becomes more specific as new knowledge is developed. Furthermore, as new entities become fully defined, they take a natural position within the classification as it currently exists, negating the need to develop a new classification system. Similarly, two closely similar entities can be shown to be specific diseases with criteria defined for distinguishing between them.

WORLD HEALTH ORGANIZATION CLASSIFICATION OF HEMATOLOGIC MALIGNANCIES

The WHO convened more than 50 pathologists with worldwide representation, as well as a large clinical advisory committee. This group largely adopted the revised European-American lymphoma classification system (REAL). They agreed that the new revised WHO Classification would replace all current systems of classification and thus brought about the first international consensus on classification of hematologic neoplasms.

Veterinarians were at first apprehensive about the application of the revised WHO system because it was suspected that a level of genetic and cytogenetic information not currently available in animal pathology would be required for application of the system. This has been a continuing factor in the diagnosis of hematologic malignancies in animals, where the large number of chromosomes in many animal species hampers cytogenetic analysis. The advent of molecular genetics has added greatly to the ability to diagnose chromosomal injuries in human medicine and has permitted animal biologists to identify chromosomal injuries in animal cells by methods such as fluorescent *in situ* hybridization (FISH).^{2,28} An advantage of the latter system is that it can be carried out on interphase cells and makes possible analysis of cells in archived paraffin blocks. The current situation is that veterinary pathologists have found that the WHO system can be applied to the great majority of animal hematologic malignancies on the basis of cell type and phenotype alone. Clonality assessment is a major current adjunct (see Chapter 143) for those cases of lymphoproliferative disease where hyperplasia or neoplasia must be differentiated.^{3,37}

A number of publications have demonstrated the use of the WHO system in animals, and remarkable similarities are being found in lymphomas of humans and animals.^{2,35,36} The more recently recognized subtypes of lymphoma in humans, including mantle cell and marginal zone lymphoma of B cell type and panniculitis-like T cell lymphoma, have now been described in domestic animals.^{9,19,35,36} Animal lymphomas are now being studied to identify molecular changes in genes that will point to their identification in humans.^{2,9} The genome of the pure bred dog is far more homogeneous than the outbred genome of humans. That reality makes the specific changes leading to tumors such as diffuse large B cell lymphoma more easily identified in dogs.

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General Features of Leukemia and Lymphoma

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Lymphoma

- Clinical Presentation
- Chemotherapy
- Assessing Remission
- Lymphoma Relapse
- Prognostic Factors

Leukemia

- Introduction
- Leukemogenesis
- Classification of Leukemias

Diagnosis of Leukemias

- Morphology
- Immunophenotyping
- Clonality assays
- Recurrent cytogenetic abnormalities

Leukemias in Dogs and Cats

- Acute leukemias
- Chronic leukemias

Acronyms and Abbreviations

ALL, acute lymphocytic (lymphoblastic) leukemia; AML, acute myelogenous (myeloid) leukemia; CBC, complete blood count; CGH, comparative genomic hybridization; CHOP, cyclophosphamide, vincristine, doxorubicin, and prednisone; CLL, chronic lymphocytic leukemia; CML, chronic myelogenous leukemia; COP, cyclophosphamide-vincristine-prednisone; CSF, colony-stimulating factor; CSFR, colony-stimulating factor receptor; FAB, French-American-British; FeLV, feline leukemia virus; FISH, fluorescent in situ hybridization; FIV, feline immunodeficiency virus; GI, gastrointestinal; GL, granular lymphocyte; HSA, hemangiosarcoma; HSC, hematopoietic stem cell; KS, Kaposi sarcoma; LSC, leukemia stem cell; MSC, mesenchymal stem cells; OSA, osteosarcoma; PCR, polymerase chain reaction; PCV, packed cell volume; REAL, revised European American classification of lymphoid neoplasms; RECIST, response evaluation criteria in solid tumors; WBC, white blood cell; WHO, World Health Organization.

LYMPHOMA

Much has been written about lymphoma in companion animals and the details of clinical presentation, diagnostics, and therapeutics are well described.^{63,64} For most affected dogs and cats, the disease is treatable but not curable. Despite initial responsiveness of most high grade lymphomas, resistance to chemotherapy, associated with progressive disease, is inevitable and is generally the reason why most dogs and cats succumb to this illness. Survival times and clinical disease-free intervals have not changed appreciably over the past 20 years despite many studies investigating new approaches to treatment. Nonetheless, chemotherapy remains the mainstay of treatment and a limited assortment of chemotherapeutic agents is widely used in practice to arrest tumor cell proliferation. Regardless of drugs used, the endpoints are almost always the same.

More recently, advances have dealt with defining prognosis for animals with malignant lymphoma. The most significant contribution in this regard is introduction and validation of the World Health Organization (WHO) classification system to veterinary oncology (see Chapter 64). This system subdivides lymphoma based on a variety of distinct histological patterns and cellular morphologies that also appear to correlate with prognosis and response to chemotherapy. Recognition of canine lymphoma as a heterogeneous malignancy has greatly advanced the clinical approach to lymphoma patients. General use of this classification system has been slow and reluctance of owners of animals with lymphoma to authorize lymph node biopsy when a diagnosis can be rendered by the less invasive and more economical fine needle aspirate has been an impediment to universal usage of this classification system. The WHO classification system may be predictive of

response to chemotherapy as well as contributing reliable information about the expected clinical course without treatment. Including the WHO classification as part of the database for evaluating new cases of lymphoma may be helpful in advising clients and selecting chemotherapeutic strategies.

Immunophenotyping lymphomas has likewise assisted with prognosis. Although it has taken nearly 20 years for this to become widely used in assessing lymphoma patients in small animal oncology,¹⁹ immunophenotyping is now widely regarded as part of the minimum database in evaluating newly diagnosed lymphoma in dogs and cats. For example, multiple studies have confirmed that approximately two-thirds of cases of multicentric lymphoma are B cell-type, with the remainder being T cell-type. Furthermore, as a general rule, dogs with B cell multicentric lymphoma have longer survival times than dogs with T cell lymphoma.³⁶ The notable exception is a form of small T cell lymphoma (T zone lymphoma) that has a better prognosis, with affected dogs surviving well past one year. Immunophenotyping feline lymphoma is less predictive of clinical outcome although there is considerable variability between immunophenotypes among the various forms of feline lymphoma.

Considered singly or in combination, cellular features (high grade versus low grade), histological classification, and immunophenotype may not always predict outcome. For example, it is possible to initiate multi-agent chemotherapy such as CHOP (i.e. cyclophosphamide, vincristine, doxorubicin, prednisone) for two dogs with identical anatomical distribution of disease (stage), identical histological classification (e.g. high grade diffuse lymphoblastic lymphoma), immunophenotype (e.g. B cell), clinical performance status (i.e. substage "a", not sick), and normal hematological and biochemical parameters, and one animal will go rapidly into complete remission and lives for 16 months while another only partially responds for 4 weeks, develops chemoresistant lymphoma, progressive disease, and survives less than 6 months. Understanding the basis for these different outcomes is one of the major challenges to improving lymphoma therapeutics. Perhaps differences in expression of oncoproteins between dogs, such as c-myc seen in some canine high-grade B cell tumors (e.g. diffuse large B cell lymphoma and Burkitt-type lymphoma) may account for these divergent clinical behaviors.¹⁶ In the case of some high-grade canine T cell lymphomas, loss of function of tumor suppressor proteins, such as the p16 cyclin-dependent kinase inhibitor that serves to regulate cellular progression through the cell cycle via the retinoblastoma (Rb) gene product, may account for different clinical outcomes between dogs that seemingly present with identical clinical, histopathological, and immunophenotypical features.¹⁶

Improving prognostic criteria for lymphoma would be of great benefit to clinicians and pet owners alike. This could assist owners in addressing difficult choices associated with treatment and end-of-life decisions. It could also move us closer to tailoring treatments to

individual patients, which may result in improved clinical outcomes, reduced client cost, and reduced treatment-associated toxicity. Discovery is key to clarifying subcellular pathologies that could account for differences in clinical outcome.

Clinical Presentation

In both dogs and cats, several distinct anatomic forms of lymphoma predominate (see Chapters 69 and 72). In the dog, the multicentric form is most commonly seen while the gastrointestinal (GI) tract is most commonly affected in the cat. Multicentric lymphoma in cats is rare. Canine multicentric lymphoma typically involves all peripheral lymph nodes and internal lymphoid tissues including tracheobronchial, sublumbar, and mesenteric lymph nodes; tonsils, spleen, and liver are also frequently involved (Fig. 65.1). The widespread use of abdominal ultrasonography has helped to demonstrate abdominal nodal involvement in many affected dogs that might have otherwise gone undetected. Thus, there is often overlap in the various anatomic presentations.

Dogs appear to have a primary gastrointestinal (GI) lymphoma in which malignant lymphocytes preferentially home to the gastric and intestinal walls and frequently infiltrate other abdominal organs including the liver, spleen, and mesenteric lymph nodes. Curiously,



FIGURE 65.1 Pitting edema of the face of a dog with multicentric lymphoma. Lymphatic obstruction caused by massively enlarged submandibular lymph nodes and a cranial mediastinal mass resulted in edema fluid accumulation throughout the dog's head.

peripheral lymph nodes seem not to be affected. This is a particularly aggressive form of lymphoma associated with a recently reported median survival time of 77 days, despite treatment with intensive chemotherapy.⁴⁸ Presumably the poor survival time is associated with inherent resistance to chemotherapy of T cell lymphoma that predominated (63%) in this study.⁴⁸

Feline alimentary lymphoma occurs as both a large cell and small cell subtype. There is considerable variability in the literature regarding immunophenotype and cell morphology. Immunophenotypical and histopathological classification of GI lymphoma in a series of 50 cats indicated that approximately half were high-grade B cell tumors and 38% were T cell lymphomas; most were small cell. Gastric lymphomas were exclusively B cell.⁴⁶ The heterogeneity of feline GI lymphoma as well as variable proliferation rates¹⁰ may account for the variability in clinical response to chemotherapeutics. More aggressive forms present with large mesenteric masses, often due to confluence of malignant mesenteric lymph nodes. Bleeding within the GI tract contributes to anemia, and bone marrow RBC regeneration may be quite slow. In the authors experience, anemia (e.g. PCV < 25%) is a negative prognostic indicator for cats newly diagnosed with GI lymphoma. Anemia in dogs (i.e. PCV < 37%) with newly diagnosed multicentric lymphoma is also a negative prognostic sign.^{1,38}

Cats have been recognized with a form of lymphoma that preferentially involves the nasal passage or nasopharyngeal tissues.^{22,31} Clinical presentation is typical of nasal cancer, mainly sneezing, nasal discharge (serous, mucoid, or blood tinged), and occasionally, facial deformity. Imaging of the skull (e.g. computed tomography) can reveal bone destruction within the head and penetration of the cribriform plate by the tumor (Fig. 65.2). Clinical staging usually fails to reveal lymphoma in other sites. The majority of these cats are affected by a high-grade immunoblastic B cell lymphoma. This form of lymphoma lends itself to treatment with radiation therapy, although multi-agent chemotherapy (alone or in combination with radiation) is also effective (median survival time = 536 days).²² Once again, anemia was determined to be a negative independent prognostic variable.

There are many other presentations of lymphoma in dogs and cats. Some are organ specific (e.g. kidney, skin, liver, central nervous system) while others involve multiple organs. With the exception of canine epitheliotropic lymphoma known to be a distinct CD3+, CD8+ T-cell disease,⁴⁰ little is known about these site-specific lymphomas.

Chemotherapy

With the exception of uniquely localized disease (e.g. nasal lymphoma), chemotherapy is the treatment of choice for most veterinary patients. As a general rule, malignant lymphoma is the most chemo-responsive cancer encountered in veterinary oncology with complete and partial remission rates of canine multicentric lymphoma reported to be in the range 75–85%.

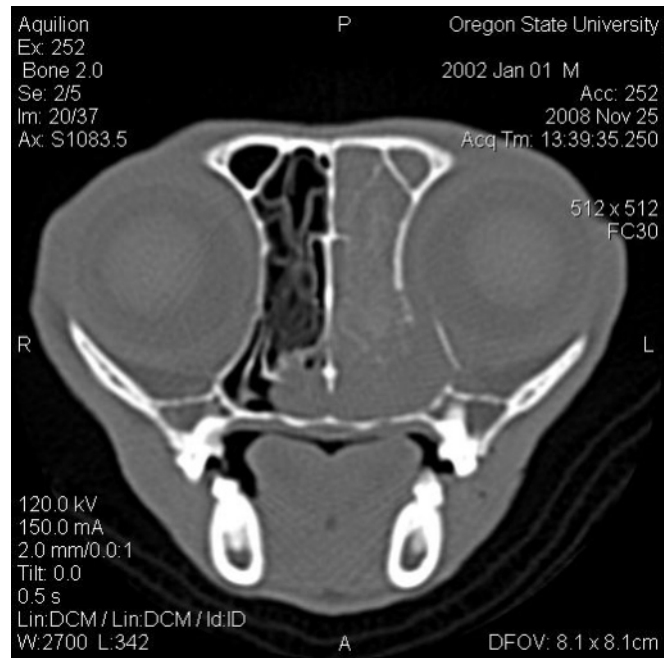


FIGURE 65.2 Computed tomography scan of a cat with intranasal lymphoma. Note the opacity filling the entire left nasal passage with extension to the right side ventrally. The medial bony orbit of the left eye has been penetrated by the tumor. This was a lymphoblastic B cell lymphoma.

Regardless of the specific protocol used to treat newly diagnosed lymphoma in dogs and cats, some common themes are apparent. High-grade lymphomas require more intensive treatment than low-grade forms and multi-agent chemotherapy provides longer and more durable remissions than does treatment with single agents. Specific protocols vary by acronyms, but usually not by agents comprising them. Most treatment protocols (e.g. CHOP, VELCAP, L-COPA) employ combinations of vincristine, cyclophosphamide, doxorubicin, and prednisone as induction agents. L-asparaginase has also been used both in canine and feline protocols but it does not appear to confer a survival advantage in treatment of canine multicentric lymphoma.³⁵ The first author prefers to reserve this drug for dogs that relapse or animals facing imminent organ failure when initially presented. More recent information would suggest that cats should receive prednisolone instead of prednisone because it has greater bioavailability in this species compared to prednisone.³³ Despite their long-standing inclusion in the treatment of lymphoma, the use of glucocorticoids arguably are the least understood drugs for treating this disease. Long thought to induce apoptosis of lymphoid cells by interaction with the glucocorticoid receptor, additional mechanisms may be important.^{52,56} Developing a better understanding of the mechanism of action and resistance may help to clarify optimum dosing schemes with prednisone/prednisolone in lymphoma chemotherapy protocols.

Despite the myriad of published protocols, the median survival time of high-grade canine lymphoma

has remained approximately 1 year. Approximately 20% of dogs will survive 2 years. Similar results were recently reported for cats treated with a cyclic multi-agent protocol,⁵⁵ although another report by Hadden et al. suggests that intensive chemotherapy (VELCAP) may not be that helpful.²⁰ The addition of doxorubicin has contributed to improved outcomes in multi-agent protocols. Taken together, these results suggest that the maximum benefit from chemotherapy has probably been attained and future improvements in the survival time of dogs and cats with high-grade lymphoma will need to come from other therapeutic modalities. Developing effective ways to overcome chemo-resistance may someday prove fruitful as well.⁸

Unlike high-grade lymphomas, dogs and cats with low-grade lymphomas do not appear to benefit by more aggressive doxorubicin-containing protocols. Presumably this is attributed to the lower mitotic index and slower growth of the malignant cells. In both species, therapy is conservative and consists of intermittent treatment with chlorambucil and predniso(lo)ne.²⁸ Clinicians routinely rely on cell morphology determined from samples obtained by fine needle aspiration. The cytological description of small cell morphology is typically inferred to indicate low-grade malignancy (which is not always the case). In the cat, small intestinal lymphoma often presents with small cell morphology and immunophenotyping confirms T cell lineage of the small cells.^{10,46,65} Typically, these cats are treated with chlorambucil and prednisolone and some survive for more than 2 years. However, the cats are not rendered free of disease and may have intermittent bouts of diarrhea. Necropsy reveals persistence of tumor cells in the submucosa, and villi are often blunted and fused accounting for chronically thin animals that do not gain weight. Nonetheless, quality of life can be excellent and owners are usually satisfied with this level of control. A case report indicated excellent response of a cat with hepatosplenic erythrophagocytic small T cell lymphoma treated with chlorambucil and prednisolone.⁹ Dogs with multicentric T zone lymphoma, considered a low grade neoplasm, treated with chlorambucil and prednisone, enjoy excellent quality of life, often for several years. Lymphadenopathy is persistent, although the first author has seen partial reduction in lymph node size with this conservative therapy after several months of treatment. Conversely, the authors have not seen clinical responses (i.e. reduction of node size) in dogs with this condition when treated with the more intensive CHOP protocol. Taken together, anecdotal evidence of a role for chlorambucil and predniso(lo)ne for treatment of chronic low-grade lymphoma in dogs and cats is supported by recent clinical reports.

Assessing Remission

Assessing therapeutic response to lymphoma therapy is not as straightforward as it would seem. Size of peripheral lymph nodes can be an easy gauge of response to treatment in dogs. However, there are no standards as to what is a big lymph node. Furthermore,

considering the large size variation in the canine population, it is easy to appreciate that a “big” lymph node in a 10kg dog may be “normal” sized in a 35kg dog. Palpation of lymph nodes is an extremely subjective means by which to evaluate response to therapy. Nevertheless, marked changes are readily appreciated while subtle ones are less clear. Caliper measurements can provide rough approximations of size, but here too, there is variability between clinicians measuring the same dog’s lymph nodes at the same time, let alone at different times. In addition, some nodes are surrounded by more fat than others further impacting the accuracy of measurements. The prescapular (i.e. caudal superficial cervical) lymph nodes are deep to the subcutis and several thin muscle layers overlay them routinely, sometimes giving them the feel of being enlarged. Caliper measurements in one dimension can be a challenge while obtaining measurements in two dimensions is harder yet. Standardizing methods for measuring lymph nodes, such as using some of the guidelines in the Response Evaluation Criteria in Solid Tumors (RECIST) criteria,¹⁵ might help to establish a uniform approach to the seemingly straightforward task of measuring lymph nodes.

Recognizing caveats associated with determining lymph node size is important in how we evaluate response to treatment. Periodic fine needle aspiration of peripheral lymph nodes for cytological evaluation is a reasonable and complementary procedure to accompany measurements. Aspirates from suspicious nodes while animals are undergoing induction chemotherapy are helpful in assessing response on a cellular level and can provide information that may indicate a need to alter treatment or indicate cytological remission. However, it must be recognized that lymph node aspirates from lymphoma dogs undergoing chemotherapy rarely look entirely normal, even when in clinical remission.

Molecular assessment of remission is perhaps the most rigorous means of detecting minimal residual disease and is orders of magnitude more sensitive than node size and light microscopy. Using a real-time polymerase chain reaction strategy. One study detected 1 lymphoma cell per 10,000 cells with primers specific for canine antigen receptor gene rearrangement of T cells and rearrangement of immunoglobulin heavy chain in B cells (see Chapter 143).⁷³ They used this approach to detect and quantify circulating lymphoma cells in the blood of dogs undergoing chemotherapy for multicentric lymphoma. While decreasing lymph node size in response to chemotherapy paralleled decreases in the number of circulating lymphoma cells, circulating lymphoma cells were detectable in all seven dogs in the study at a time when the dogs were considered to be in clinical remission by conventional means.

Assessing remission in tissues that are not readily accessible for repeated sampling such as the intestinal tract, presents other unique challenges. Intestinal lymphoma is often a diffuse disease that involves large segments of the GI tract. Repetitive abdominal ultrasonographic examinations are helpful in evaluating

mural thickness and layering of the intestines, but repeated surgical or endoscopic intestinal biopsies are rarely done. Ultrasound guided fine needle aspiration of mesenteric lymph nodes is helpful, but rigorous ante mortem methods, other than biopsy, that unequivocally clarify remission status of GI lymphoma are not available. Instead, clinicians usually rely on surrogate readouts of response such as resolution of GI signs, appetite, weight gain, and improved hematocrit to aid in assessing remission. Serum cobalamin concentrations of cats with GI lymphoma are often low²⁸ at diagnosis, presumably due to malabsorption; repeated measurements could also be helpful to assess improvement in gut function in response to therapy.

Lymphoma Relapse

For most dogs and cats with lymphoma, relapse is inevitable. Animals enjoying complete clinical remission still harbor malignant cells. Why chemotherapy that is so effective in reducing tumor burden is ineffective in curing the majority of lymphoma patients awaits clarification. Innate resistance, predicted by the Goldie-Coldman hypothesis, acquired resistance associated with upregulation of cellular transporters such as p-glycoprotein,³ and uniquely resistant cancer stem cells may in part help to explain the failure of chemotherapy to be curative for most animals in veterinary oncology. Most animals have excellent quality of life at the time of relapse so it is difficult for owners and clinicians to just give up when relapse is detected.

More is known about responses in relapsed dogs than cats. Regardless of drugs used to treat relapsed canine lymphoma patients, we know that responses are not as robust or durable compared to those obtained when treating newly diagnosed patients. While there are a number of choices available for second induction chemotherapy (also known as rescue or salvage chemotherapy), most approaches fail to control relapsed lymphoma for longer than 2–4 months. Typically, when an animal fails one rescue protocol, another is offered, often based on clinician's bias. In the end, it probably does not make a difference which drugs are used to treat relapsed lymphoma patients, which raises the question, Does rescue chemotherapy really rescue?

In the first author's experience, animals deemed to be in complete remission (i.e. >3 months following completion of CHOP induction) will likely respond to the protocol again, albeit for shorter duration. Keep in mind that doxorubicin use is limited in the rescue setting due to its cumulative cardiotoxic effects. Sometimes at the first author's institution, an abbreviated course of CHOP (e.g. eight treatments) is administered in the rescue setting. L-asparaginase is very effective for short-term gains, especially as a "quick fix" to cytoreduce relapsed lymphoma when owners may need time to consider their options or when a dog is being adversely affected by the relapse (e.g. airway obstruction by lymphomatous tonsils, etc.). Lomustine has become widely used in the relapse setting based on the report by Moore

et al. over 10 years ago.⁴¹ Given orally as a capsule, it is simple and appealing to owners. However, duration of response is less than 3 months and it can have a profoundly suppressive effect on bone marrow. The dosage used in the initial report was high (90 mg/m²), a dosage that many have found to be toxic (typical starting dosage is 60 mg/m²) and the benefit of lomustine is relatively short-lived in dogs. In an effort to enhance lomustine's efficacy, Saba et al. combined lomustine with L-asparaginase concurrently hoping to obtain additive (or synergistic) benefits from the combination.⁵¹ The median time to disease progression was 63 days indicating the combination did not confer an advantage over lomustine alone. Based on this result it is probably reasonable to separate administration of these two drugs with L-asparaginase given first considering its typically rapid response, followed by lomustine 1–2 weeks later. A plethora of drugs or drug combinations have been used to treat relapsed lymphoma, including mechlorethamine, procarbazine, actinomycin-D, cisplatin, carbaplatin, dacarbazine, etoposide, and bleomycin to name a few. None have distinguished themselves as the antidote for relapsed lymphoma. New approaches are needed for relapsed lymphoma.

Hematopoietic stem cell transfer (also known as bone marrow transplant) has been on the fringes of veterinary oncology for many years. Despite the use of research dogs for the development of this procedure in human oncology, the expense, support, and need for expertise in tissue typing have impeded implementation of this procedure in veterinary oncology. Nonetheless, the recent case report by Lupu et al. of a successful and seemingly curative allogeneic hematopoietic stem cell transplant in a golden retriever with relapsed T cell lymphoma has renewed enthusiasm amongst oncologists.³⁴ As of this writing, an autologous bone marrow transplant program for dogs with lymphoma has been established at North Carolina State University.

A promising approach for human B cell lymphoma employs a monoclonal antibody (rituximab) that binds to CD20 on the surface of human B cells. Once bound, the antibody can mediate antibody-dependent cellular cytotoxicity or it can be armed with an isotope or toxin for targeted delivery. Impressive results with these approaches have been published. While normal and malignant canine B cells express CD20,²⁷ disappointingly, rituximab failed to bind or deplete canine B cells.^{25,27} Developing CD20 as a therapeutic target in canine B cell lymphoma will require identification or generation of unique antibodies that recognize the extracellular domain of this homolog in the dog.

Prognostic Factors

Lymphoma is a heterogeneous disease and treatment outcomes can differ widely between animals that present with similar clinical features. Thus, identifying factors that predict outcome is important in setting expectations for pet owners as well as clinicians. More

is known about prognostic features in canine lymphoma than for feline lymphoma. In dogs, the list of prognostic variables has slowly increased. Immunophenotype remains one of the strongest prognostic variables. Degree of cellular differentiation is also prognostic. Dogs with $\geq 50\%$ bone marrow infiltration by lymphoma cells (stage V) are predicted to do poorly even with chemotherapy. Clinical illness at initial presentation (i.e. substage "b") is likewise a negative predictive feature, as is anemia.³⁸ High expression of the anti-apoptotic protein, survivin, predicts early relapse in dogs with stage IIIa or IVa B cell lymphoma.⁴⁹ In contrast to dogs, immunophenotype has not been predictive of outcome in cats, but anemia in cats with nasal lymphoma is a negative prognostic factor. Recognizing features and biomarkers that directly correlate with clinical outcome enhances our understanding of lymphoma and provides a framework to explain different outcomes between affected animals.

LEUKEMIA

Leukemias are a group of neoplastic diseases, described in dogs, cats, horses, cattle, humans, and a variety of other species, characterized by the clonal proliferation of malignant hematopoietic progenitor cells in the bone marrow. Leukemias are broadly categorized as being of either lymphoid or myeloid origin. Myeloid neoplasms are further classified by cellular lineage into four major categories including granulocytic (neutrophils, eosinophils, and basophils), monocytic, erythroid, and megakaryocytic. Occasionally, myeloid leukemias may be of mixed-cell lineage. Both lymphoid and myeloid leukemias are further divided into acute and chronic types based on clinical presentation and proliferative rate of the neoplastic clone.

Leukemogenesis

Leukemias are thought to represent an aberrant hematopoietic process initiated by rare leukemia stem cells (LSCs) that have maintained or reacquired the capacity for indefinite proliferation through accumulated mutations and/or epigenetic changes. In normal hematopoiesis (Section I), stepwise differentiation of hematopoietic stem cells (HSCs) generates a hierarchy of progenitor populations, each with a progressively restricted developmental potential, ultimately producing all lineages of mature blood cells. In contrast to HSCs, which can self-renew for life, multipotent myeloid and lymphoid progenitors do not have self-renewal capacity. Similarly, leukemic stem cells may have limitless self-renewal capacity and give rise to clonogenic leukemic progenitors. These cells may not have self-renewal capacity but are incapable of normal hematopoietic differentiation.⁴³ Experimentally, leukemias may be initiated by transforming events that take place in the HSC population, or from more committed progenitors owing to mutations, epigenetic changes, and/or selective gene expression which enhances their otherwise limited self-

renewal capabilities. In fact, deregulation of self-renewal pathways appears to be a common mechanism used by acute myeloid leukemia (AML)-associated fusion genes to transform both HSC and progenitor cell populations.^{43,44}

The understanding of the molecular basis of leukemia is the most advanced for chronic myelogenous leukemia (CML). Based on the molecular pathogenesis of CML in humans, it has been suggested that leukemias in general result from mutations in genes that encode key molecules that dysregulate the normal strict cytokine/receptor dependent control of the decision of hematopoietic cells to undergo proliferative expansion.⁵⁷ For a leukemia to develop, a selective growth advantage must occur in a single mutated cell (typically a rare HSC, or, if self-renewal capabilities are restored, a more committed progenitor), thereby allowing its progeny to undergo proliferative expansion, which ultimately leads to accumulation and dissemination of leukemia cells.^{43,44} In the case of human CML, the BCR-ABL mutation provides that selective growth advantage by perturbing the cytokine/receptor/JAK/STAT/cyclin D pathway, proliferative expansion, and subsequent accumulation of multiple additional mutations and blast transformation.⁵⁷

Classification of Leukemias

The pathological classification used to describe lymphoid and myeloid leukemias in animals is the current WHO⁶⁷ classification system that is modified from the human French-American-British (FAB)² and REAL systems²³ (see Chapter 64). The FAB system has been applied previously to animal hematopathology.²⁶ An important feature of the REAL/WHO classification system is that it attempts to define specific disease entities (i.e. not just different histopathological diagnoses based on morphology alone). Nonetheless, in some cases, proposed pathologically defined disease entities lack good clinical descriptions as uniquely defined clinical entities. However, this classification system also recognizes that essentially the same disease may present clinically in different forms. For example, in this system, it is recognized that the distinction between leukemia and lymphoma in some cases may be imperfect and merely represents a continuum of clinical presentation of the same disease.⁴ In this case, the diagnostic distinction is based on determining the tissue with the greatest volume of tumor:⁶⁶ if most of the neoplasm is in the bone marrow it is termed leukemia, and if most is in peripheral tissues it is termed lymphoma.

Diagnosis of Leukemias

The initial clinical presentation of an animal with leukemia varies; however, as the names suggest, animals with acute leukemias usually present initially with acute systemic signs, whereas those with chronic leukemias often present with chronic, less severe clinical signs. Initial evaluation of an animal with leukemia

starts with a physical examination and complete blood count (CBC). Once a presumptive diagnosis of leukemia is established, based upon the physical examination and CBC findings, a minimum database including a serum biochemical profile, urinalysis, and bone marrow aspirate and/or core biopsy should be obtained. Diagnostic imaging of the patient provides additional information regarding involvement of other organs.

Morphology

Until relatively recently, a diagnosis of leukemia was based primarily on cytological examination of blood smears, and cytological and histological morphology of bone marrow aspirates and core biopsies, respectively, from affected animals. The subsequent development of enzyme cytochemistry, immunophenotyping techniques, clonality assays, gene expression profiling, and assessment of chromosomal aberrations, have illustrated that, although useful, and even necessary, as a first level of evaluation, morphology alone is insufficient for correctly classifying many leukemias.⁶⁸ Information gained through these additional techniques has been incorporated into the REAL/WHO classification system.

The initial diagnosis of leukemia is usually made based on morphological assessment of a peripheral blood smear and bone marrow aspirate and/or core biopsy (see Chapter 132). Neoplastic cells are frequently identified in the blood smear, although aleukemic leukemias occur rarely. Identification of malignant hematopoietic cells in the blood and bone marrow can usually be made based on morphology alone, however further classification of the leukemia is often misleading or impossible based on morphology alone.

Immunophenotyping

Flow cytometry (see Chapter 137), used in conjunction with a panel of cell surface markers, can be used to identify and characterize malignant cell populations in blood and bone marrow.^{17,68,70} The immunophenotype of leukemic cells provides information regarding the presumptive cell lineage, a critical component to correct classification (see Chapters 4 and 144). The antibodies available for immunophenotyping hematopoietic cells in dogs, cats, and other domestic animals are more limited than in humans and mice. Table 65.1 is an example of a diagnostic panel of antibodies that can be used to immunophenotype canine leukemias. Unfortunately, there remains a paucity of validated myeloid lineage markers, so subclassification of myeloid leukemias is difficult.

Clonality Assays

Leukemias generally are considered to be clonal or oligoclonal diseases; therefore, demonstration of a clonal population of cells in the blood or bone marrow is supportive of a diagnosis of leukemia. Clonality of lym-

TABLE 65.1 Monoclonal Antibodies used for Immunophenotyping Canine Leukemias and Lymphomas by Flow Cytometry and Immunocytochemistry

Antigen	Clone
IgG1 (controls)	W3/25
CD1a	CA9.AG5
CD1c	CA13.9H11
CD3	CA 17.2 A12
CD3e	CD3-12
CD4	YKIX 302.9
CD4	CA13.1E4
CD5	YKIX 322.3
CD8	YCATE 55.9
CD8	CA9.JD3
CD8	CA15.4G2
CD11a	CA11.4D3
CD11a	CA16.1B11
CD11b	CA16.3E10
CD11c	CA11.6A1
CD11d	CA11.8H2
CD14	TUK 4
CD18	YFC 118.3
CD18	CA1.4E9
CD20	polyclonal
CD21	B-ly4
CD21	CA2.1D6
CD22	RFB4
CD34	1 H6
CD45	YKIX 716.13
CD45	CA12.10C12
CD45RA	CA4.1D3
CD49-like (VLA-4)	CA4.5B3
CD54 (ICAM-1)	CL18.1D8
CD56	B159
CD79a (MB-1)	HM57
CD90 (Thy-1)	CA1.4G8
CD117 (c-Kit)	YB5.88
MHC I	H58A
MHC II	YKIX 3342
MHC II	CA2.1C12
TCR $\alpha\beta$	CA15.8G7
TCR $\gamma\delta$	CA20.8H1
TCR $\gamma\delta$	CA20.6A3
MPO	CA25.10A6
Mac387 (L1-calprotectin)	Polyclonal

phoid cell populations can be determined using PCR-based assays for immunoglobulin and T cell receptor gene rearrangements (see Chapter 143).⁷ A similar diagnostic test is not available for demonstrating clonality for myeloid leukemias. A negative clonality test for immunoglobulin or T cell receptor gene rearrangement is consistent with, but not diagnostic for, myeloid leukemia.

Recurrent Cytogenetic Abnormalities

In humans, the WHO classification recognizes certain leukemias with recurrent cytogenetic abnormalities as

distinct disease entities. Recent studies using standard cytogenetic, fluorescent in situ hybridization (FISH), comparative genomic hybridization (CGH), and other molecular and genomic techniques have begun to identify recurrent cytogenetic abnormalities in canine lymphomas and leukemias (see Chapter 148).^{21,47,61,62}

Leukemias in Dogs and Cats

Leukemias are rare in dogs and cats however their true incidence is unknown. Although most leukemias are considered to be spontaneous in origin, the association of specific leukemia/lymphoma subtypes with certain breeds of dogs and identification of recurrent chromosomal aberrations indicate that a heritable component contributes to the pathogenesis of leukemia in this species.⁶ Leukemias have been induced in dogs with experimental radiation exposure³⁹ and certain gene therapy vectors.^{53,74} Viral particles have been identified in leukemic cells in a few instances,^{18,45,74} but a clear role for a virus in leukemia pathogenesis has not been demonstrated. In the cat, both lymphoid and myeloid leukemias have been associated with feline leukemia virus (FeLV; see Chapter 62)⁵⁰ and myeloid leukemias with feline immunodeficiency virus (FIV) infection (see Chapter 55).^{24,54}

Acute Leukemias

Acute lymphocytic (lymphoblastic) leukemia (ALL) is a clonal proliferation of malignant lymphoid progenitors in the bone marrow. Cells show little differentiation. Blasts and prolymphocytes are the predominant cell population. Acute myelogenous leukemias (AMLs) are neoplastic myeloproliferative disorders originating from hematopoietic stem cells that differentiate to granulocytic, monocytic, erythrocytic, and megakaryocytic lineages. Acute myelogenous leukemias are characterized by greater than 20% blasts in the bone marrow, with an elevated white blood cell (WBC) count and high numbers of circulating atypical blasts.

In animals, acute leukemias can occur in most domestic and exotic species; however, it is probably most significant as a clinical entity in dogs and cats. Bovine leukemia complex (see Chapter 62) is an important retroviral-induced tumor.⁴² The incidence of acute leukemias in dogs and cats is uncertain, although it is generally thought to be less common than lymphoma. Historically, AML in the dog has been reported to be more common than ALL. In two recent studies where immunophenotyping was performed, AML was more frequent;^{69,70} AMLs comprised 21 of 38 (58%) and 31 of 46 (67%) of acute leukemias, respectively. However, the true frequency of AML versus ALL is unresolved given the case selection biases in these studies.

Lymphoid leukemia is reported to be the most common leukemia in cats, with ALL the most common lymphoid leukemia.¹² Acute lymphocytic leukemia in the cat had been highly correlated with FeLV infection, although this may be changing. Myelomonocytic

(M4) is the most common acute myeloid leukemia reported in the cat.⁵ With improved control of retroviral diseases in cats, there has been an apparent decrease in the incidence of FeLV-associated tumors in cats.³² Immunophenotyping of a large series of acute leukemias in cats has not been reported.

The FAB cooperative group classification system has been applied to the classification of acute leukemias in the dog and cat²⁶ and has been incorporated into the REAL/WHO classification system. For the most part, detailed clinical description of many of the pathologically defined disease entities is limited. Until relatively recently, enzyme cytochemical analysis was the primary method used to identify the origin (i.e. cell lineage) of hematopoietic tumor cells. Immunophenotyping of leukemia, primarily using flow cytometry, has largely replaced enzyme cytochemical staining for diagnosing and classifying leukemias in cats and dogs (although cytochemical stains may provide confirmatory, or additional diagnostic information). In people, the presence of specific recurrent cytogenetic abnormalities is being incorporated into the WHO classification system and in some cases is being used to define specific leukemia diagnoses.^{59,60}

The clinical signs and physical examination findings in dogs with acute leukemia may be vague and nonspecific.¹³ In contrast to most dogs with lymphoma, dogs with acute leukemia usually are clinically ill at initial presentation. Dogs may present with anorexia, lethargy, fever, weight loss, shifting limb lameness, or neurologic signs. On physical examination splenomegaly, hepatomegaly, pale mucous membranes, petechia or ecchymosis, and mild generalized lymphadenopathy are common findings. Shifting limb lameness, fever, and ocular lesions have been reported to be more common in AML, and neurologic signs are reportedly more common in ALL.¹³

Neoplastic (leukemic) cells are present in the peripheral blood of most dogs with acute leukemia. The total WBC count tends to be higher in ALL. Acute leukemias with WBC counts greater than 150,000/ μ L, are generally ALL. Almost all dogs with acute leukemia have mono-, bi-, or pancytopenias present. Most affected dogs are anemic and thrombocytopenic.¹³

A diagnosis of acute leukemia can usually be made based on the history, physical examination findings, and CBC. Dogs with aleukemic leukemia require a bone marrow biopsy or aspirate to confirm the diagnosis. Greater than 20% blasts present in the bone marrow is consistent with a diagnosis of leukemia. Further diagnostic testing to assess the patient and determine the extent of disease includes abdominal ultrasound with lymph node, liver, and splenic aspirates. Flow cytometry of bone marrow aspirates and/or blood are required to determine immunophenotype. It is well documented that morphology alone is insufficient for distinguishing lymphoid from myeloid leukemia.⁷⁰

Distinguishing acute leukemia from high-grade lymphoma in dogs with mild lymphadenopathy, significant bone marrow involvement (WHO clinical stage V), and a low number of circulating blasts, also can be dif-

ficult. In such cases, dogs that are systemically ill, have bicytopenia or pancytopenia, or a high percentage of blasts in bone marrow, are more likely to have ALL.¹³ Immunophenotyping also provides valuable information to make the distinction of lymphoma versus leukemia. CD34 is usually expressed in acute leukemias, whereas lymphomas are CD34-negative greater than 70% of the time.^{70,71}

Cats with acute leukemias usually show similar clinical signs (pale mucous membranes, splenomegaly, fever). Leukemic cats are likely to have malignant cells infiltrating in other organs besides bone marrow.⁵ All cats with leukemia should be tested for FeLV and FIV. Treatment of ALL in cats is disappointing. A 27% complete response rate and median remission of 7 months was reported using a COP chemotherapy protocol.¹¹ Intensive supportive therapy, including blood transfusions, antibiotics, and nutritional support is frequently needed.

Determination of the specific type of leukemia has potential prognostic and therapeutic implications in dogs and cats; however, in general, both ALL and AML have a very poor prognosis. Remissions are usually difficult to achieve and survival times are short, even with treatment.¹⁴ Treatment of acute leukemias has, for the most part, been extrapolated from chemotherapy protocols used to treat people with what appear to be similar diseases. Treatment of acute leukemias in dogs and cats has not been rigorously evaluated. Only retrospective data have been reported. These animals usually require intensive supportive treatment. More than half of dogs treated for acute leukemia die of sepsis or bleeding.^{13,14} ALL is typically treated with the same combination and sequential chemotherapy protocols as is used to treat lymphoma, with doxorubicin-based protocols generally being preferred. Remission rates are considerably lower than those observed for solid lymphomas (20–40%). Survival times are also shorter, averaging 1–3 months.^{13,14} The prognosis for animals with AML is particularly poor: most animals die or are euthanized shortly after diagnosis. Treatment usually consists of cytosine arabinoside, either as a single agent, or combined with other chemotherapeutics.¹⁴ Remissions are rare, and when they occur short-lived.

Chronic Leukemias

Chronic lymphocytic leukemia (CLL) is a neoplastic clonal proliferation of small and morphologically mature-appearing lymphocytes that manifests as a persistent (>3 months), often marked, peripheral lymphocytosis.⁷² Anecdotally, it is thought to be the most common leukemia in cats and dogs, but the true incidence is unknown. In the dog, CLL is seen most commonly in middle-aged to older dogs, with a reported median age of about 10 years, and a range of 1.5–15 years.⁷² CLL has been reported in many different breeds and mixed breed dogs. In one study, golden retrievers and German shepherd dogs were reported to be over-represented.³⁷ CLL occurs in cats; however, beyond case

reports, little has been published. Anecdotally, CLL appears to occur primarily in older cats, with an approximate mean age of 12.2 years and a range of 6–19 years (W. Vernau, personal communication). The cause of CLL in animals is unknown, but for the most part has not been studied. Recently, Breen and Modiano showed hemizygous or homozygous deletion of the *RB1* locus (including the microRNA cluster encoding mir15 and mir16) in five of seven cases of canine CLL, with associated loss or reduction of RB protein in nine of ten cases.⁶ This was reported to be homologous to the common deletion in the q arm of chromosome 13 (13q14) seen in people with CLL.

CLL is a heterogeneous disease, in the sense that it can involve proliferation of morphologically mature members of any of the different lymphocyte subsets. The clinical presentation of CLL in dogs and cats is variable; however, most commonly animals are asymptomatic at initial diagnosis. In fact, the initial suspicion of CLL frequently is based on an incidental finding of lymphocytosis found during a geriatric wellness examination or before planned surgical procedure. Clinical signs are often vague and nonspecific, including lethargy, reduced appetite, and slowly progressive weight loss.⁷² Physical examination findings may be unremarkable, or include mild generalized lymphadenopathy, splenomegaly, hepatomegaly, pale mucous membranes, and pyrexia.⁷²

The primary laboratory finding in CLL is leukocytosis caused by an absolute lymphocytosis. Lymphocytosis can range from mild to marked and can fluctuate, even without therapy. Reported lymphocyte counts in dogs with CLL have been reported as varying from just outside the laboratory normal range to over 1,000,000/ μ L.^{37,72} In the cat, lymphocyte counts varying from 36,000 to 250,000/ μ L have been reported. Other hematological findings may include normocytic normochromic nonregenerative anemia in greater than 50% of affected dogs, and thrombocytopenia in 25–50% of dogs with CLL. Neutropenia is rarely observed. In cats, hematological findings included anemia (61%), thrombocytopenia (38%), neutropenia (16%), and neutrophilia (50%). Monoclonal gammopathies are reported in approximately two-thirds of dogs with CLL. That is a paradoxical finding, given that the majority of CLLs in dogs are T cell neoplasms. Other paraneoplastic syndromes that may occur include immune-mediated hemolytic anemia, pure red cell aplasia, and hypercalcemia.

B cell CLL in the dog, as is the case in people, is a primary bone marrow disease. Similar to people, dogs with B cell CLL typically have significant (>30%) proliferation of small mature lymphocytes in bone marrow.⁷² In contrast, canine T cell CLL of the large granular lymphocyte type (GL CLL) does not seem to be a primary bone marrow disease, but rather a primary splenic disease.^{37,72} A similar splenic origin for large GL leukemia is well-characterized in Fischer 344 rats.⁵⁸ Feline CLL appears to be primarily a helper T cell disease, although other T cell immunophenotypes occur (W. Vernau, personal communication).

Absolute lymphocytosis is the major diagnostic criterion for CLL in dogs. Other differential diagnoses for dogs with mild lymphocytosis (7,000–20,000/ μL) include ehrlichiosis, babesiosis, leishmaniasis, Rocky Mountain spotted fever, Chagas' disease, hypoadrenocorticism, and physiologic leukocytosis. Lymphocytosis ($>20,000/\mu\text{L}$ in the dog or $>25,000/\mu\text{L}$ in the cat) is consistent with CLL. In those cases where the differentiation of CLL from other diseases is problematic, immunophenotyping and clonality assessment can be useful.

In people, B cell CLL (CD5+, CD79a+, CD21+), involving proliferation of the CD5+ B-1 subset of B cells, accounts for more than 95% of CLL cases. In contrast, in the dog, T cell CLL appears to be far more common than B cell CLL.^{68,72} In the largest reported study of 73 dogs with immunophenotyped CLL, 73% of cases involved proliferation of T cells and 74% of those had GL morphology. Most GL CLLs had a cytotoxic T cell immunophenotype (CD3+, CD8+, CD11d+). B cell CLL comprised 26% of canine CLL cases in this study, based on expression of CD21 or CD79a and lack of expression of any T cell antigens.⁶⁸ In cats with CLL, the predominant immunophenotype appears to be that of the T helper cell (CD3+, CD4+, CD5+), accounting for 75% of cases. Cytotoxic T cell (CD3+, CD8+, CD5+), double-positive (CD4+, CD8+), and double negative (CD4-, CD8-) immunophenotypes also can occur. In situations where CLL is the primary differential diagnosis, but where doubt still exists, assessment of clonality by PCR can be particularly useful.

CLL in the dog is generally indolent; however, prospective studies with regular and close clinical follow-up are lacking. While the disease has been generally perceived to inexorably progress to blast transformation, a recent study showed that dogs with absolute lymphocyte counts of $<30,000/\mu\text{L}$ had prolonged survival with conservative management.⁷¹ In the authors' experience, uncomplicated (lymphocytosis without other cytopenias, no or mild anemia, minimal organ involvement) CLL is indeed indolent. With treatment, dogs with uncomplicated CLL may live more than 3 years with their disease.^{29,68,72} However, survival times can be quite short in complicated (lymphocytosis, moderate or severe anemia, significant organ involvement, presence of paraneoplastic syndromes, poor clinical response to chemotherapy) CLL, so it is appropriate to describe CLL as a heterogeneous disease with realistic survival expectations from ~1 to 3 years.²⁹ The prognostic significance of CLL immunophenotype has only begun to be investigated. In the largest published retrospective study, immunophenotype was not correlated with prognosis.⁶⁸

Because CLL is often an indolent disease, approaches to treatment vary. Some oncologists prefer to manage asymptomatic dogs with mild to moderate lymphocytosis using "watchful waiting," to monitor for progression, an approach that is similar to the clinical management for people with CLL. In cases where there is progression (defined by a persistent trend of increas-

ing absolute lymphocyte counts in peripheral blood or appearance of other systemic signs), management with chemotherapy is instituted. In the authors' practices, most dogs and cats with CLL are managed with chemotherapy at the onset of diagnosis. Dogs are generally treated with pulse dose chlorambucil at a dosage of 20 mg/ m^2 orally once every 14 days.¹⁴ Corticosteroids, prednisone, at a dosage of 50 mg/ m^2 orally once daily for 7 days, then 25 mg/ m^2 orally every other day, may be included, particularly if paraneoplastic syndromes are present. Responses to treatment are typically slow, probably because the growth fraction of neoplastic lymphocytes in CLL appears to be low. Generally, it takes at least a month of treatment to observe a response to therapy and may take as long as 6 months.¹⁴ A consensus on the best approach to manage uncomplicated CLL will require prospective assessment of observation versus therapy in a controlled setting.

Chronic myeloid leukemia (CML) is extremely rare and is poorly characterized in dogs and cats.³⁰ The chronic myeloid leukemias include chronic myelogenous or granulocytic, chronic monocytic, and chronic myelomonocytic leukemias. The clinical signs and physical examination findings in dogs and cats with CML appear to be similar to those in dogs and cats with CLL. Blast crisis, manifested by the appearance of immature blast cells in blood and bone marrow, occurs in dogs and humans with CML months to years after initial diagnosis. Blast crisis appears to be rare in dogs with CML. The hematological features of CML in dogs are poorly characterized. Leukocytosis with a left shift that includes myelocytes and occasional myeloblasts, anemia, and thrombocytopenia or thrombocytosis, can occur. Typically, mature forms are more numerous, but are left-shifted. Signs of dysplasia may be present, including hypersegmentation, ringed nuclei, and giant forms. The diagnosis of CML in the dog and cat is challenging, particularly because the disease is so poorly characterized. Other differential diagnoses include severe neutrophilic leukocytosis and leukemoid reactions caused by inflammation or immune-mediated disease.

In humans, CML is characterized by the presence of a recurrent chromosomal aberration, referred to as the Philadelphia chromosome, which represents a reciprocal translocation between the *c-abl* oncogene located in 9q34 (*ABL* locus) and the breakpoint cluster region (BCR) located at 22q11. Recently, a homologous translocation was demonstrated to occur in dogs with spontaneously occurring CML.⁶ The prevalence of this translocation in canine CML has yet to be determined. Treatment of CML in dogs with hydroxyurea has been extrapolated from the human literature. Based on anecdotal and case reports, the prognosis does not appear to be as good as that for dogs with CLL, with reported survival times ranging from 4 to 15 months. Presumably, the tyrosine kinase inhibitors imatinib (Gleevec) and sunitinib (Stentent), and the new tyrosine kinase inhibitors developed for the veterinary market, toceranib phosphate (Palladia) and masitinib (Kinavet), may be

effective in animals with dysregulated Abl protein expression. However, there are no verified reports of their efficacy to treat canine or feline CML.

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Myelodysplastic Syndromes

DOUGLAS J. WEISS

Classification of Myelodysplastic Syndromes

Myelodysplastic Syndromes in Dogs

Refractory Anemia

Refractory Anemia with Ringed Sideroblasts

Refractory Cytopenia with Multilineage Dysplasia

Refractory Anemia with Excess Blasts

Myelodysplastic Syndrome Unclassifiable

Secondary Myelodysplastic Syndromes

Secondary Dysmyelopoiesis in Dogs

Diseases/Drugs/Toxins

Congenital Dysmyelopoiesis (see Chapter 29)

Myelodysplastic Syndromes in Cats

Refractory Anemia

Refractory Anemia with Ringed Sideroblasts

Refractory Cytopenia with Multilineage Dysplasia

Refractory Anemia with Excess Blasts

Secondary Dysmyelopoiesis in Cats

Diseases/Drugs/Toxins

Myelodysplastic Syndrome in Horses

Acronyms and Abbreviations

DNA, deoxyribonucleic acid; FeLV, feline leukemia virus; MCV, mean corpuscular volume; MDS, myelodysplastic syndrome; RA, refractory anemia; RAEB, refractory anemia with excess myeloblasts; RAMD, refractory anemia with excess myeloblasts; RARS, refractory anemia with ringed sideroblasts; RBC, red blood cell; RCMD, refractory cytopenia with multilineage dysplasia; WHO, World Health Organization.

Myelodysplastic syndromes (MDSs) are a group of acquired clonal hematologic disorders, described in dogs, cats, horses, and humans, that originate from acquired genetic mutations in hematopoietic stem cells.^{3,5,8,13,41} A clonal origin of MDS has been documented in human beings by homozygosity for glucose-6-phosphatase isoenzymes, cytogenetic analysis, and X-linked restriction length polymorphism.^{2,16} A clonal origin has been documented in cats infected with feline leukemia virus (FeLV).¹⁶ Myelodysplastic syndromes in dogs has been assumed to be clonal because of the lack of concurrent diseases or drug or toxin exposure, and because of the similarity of these disorders to those described in humans.^{21,41} As in other neoplasms, clonal expansion is usually the result of multiple mutations in oncogenes and tumor suppresser genes (see Chapter 61).^{2,16} These syndromes are heterogeneous but are characterized by cytopenias in the blood and dysplastic changes in one or more cell lineages in bone marrow. Cytopenias result from a combination of defects in growth and differentiation and increased apoptosis of hematopoietic cells. These defects result in ineffective hematopoiesis.^{5,22,28} Myelodysplastic

syndromes are highly heterogeneous and major subtypes must be differentiated in order to determine appropriate treatment and to establish a prognosis. Myelodysplastic syndromes must also be differentiated from dysmyelopoiesis occurring secondary to diseases, drug and toxin exposure, and as a result of congenital disorders.^{1,15,32,35,38} The most reliable features that help to differentiate MDS from secondary dysmyelopoiesis include the presence of increased numbers of myeloblasts or rubriblasts in bone marrow, a high percentage of dysplastic cells in bone marrow, and the presence of prominent megaloblastic changes in erythroid precursor cells.^{5,38} Congenital dysmyelopoiesis is generally seen in juvenile animals and may be breed-restricted (see Chapter 30).

CLASSIFICATION OF MYELODYSPLASTIC SYNDROMES

Myelodysplastic syndromes were initially classified by the French-American-British co-operative group (Table 66.1; see Chapter 64).⁴ This system has been applied to

both dogs and cats.⁵ The French-American-British system categorized MDS into five categories primarily based on the distribution and type of dysplastic features and the percentage of myeloblasts present. These five categories included refractory anemia (RA), refractory anemia with ringed sideroblasts (RARS), refractory anemia with excess blasts (RAEB), refractory anemia with excess blasts in transition (i.e. 20–30% myeloblasts in bone marrow), and chronic myelomonocytic leukemia. Acute leukemia was defined as greater than 30% myeloblasts in bone marrow.⁴ More recently, the World Health Organization (WHO) modified the French-American-British classification system incorporating some new diagnostic information including cytogenetics, immunologic markers, and molecular genetics.¹³ In the WHO classification system, RA is defined as nonre-

generative anemia with dysplastic changes primarily in the erythroid lineage and less than 5% myeloblasts in bone marrow (Figs. 66.1 and 66.2). Refractory anemia with ringed sideroblasts is defined as dyserythropoiesis characterized by siderocytes, sideroblasts, and ringed sideroblasts, and with dysplastic features primarily in the erythroid lineage and less than 5% myeloblasts in bone marrow. Sideroblasts are nucleated erythroid cells that contain many large iron deposits within mitochondria. Because mitochondria are distributed around the nucleus, iron deposits frequently form a perinuclear collar and the cells are termed ringed sideroblasts (Fig. 66.3). Refractory cytopenia with multilineage dysplasia (RCMD) is characterized by dysplastic changes in all hematopoietic cell lineages but no increase in the number of myeloblasts in bone marrow. Refractory

TABLE 66.1 Classification Systems for Myelodysplastic Syndromes

French-American-British	World Health Organization	Dog/Cat System
Refractory anemia	Refractory anemia	MDS with refractory cytopenia
Refractory anemia with ringed sideroblasts	Refractory anemia with ringed sideroblasts Refractory cytopenias with multilineage dysplasia	MDS with sideroblastic differentiation
Refractory anemia with excess blasts	Refractory anemia with excess blasts-1 (5–10% blasts)	MDS with excess blasts (5–30% blasts)
Refractory anemia with excess blasts in transition	Refractory anemia with excess blasts-2 (10–20% blasts) MDS with isolated del(5q) chromosome abnormality MDS unclassifiable	MDS unclassifiable
Chronic myelomonocytic leukemia		
Acute myeloid leukemia (>30% blasts)	Acute myeloid leukemia (>20% blasts)	Acute myeloid leukemia (>30% blasts)

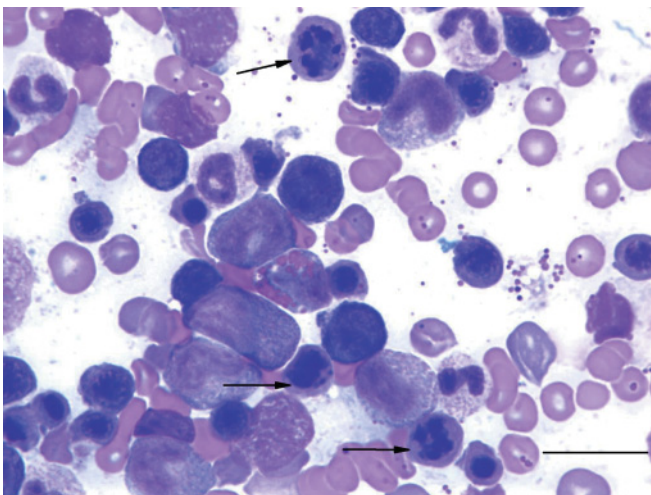


FIGURE 66.1 Dyserythropoiesis in a bone marrow aspiration smear from a dog with refractory anemia. Note the presence of fragmented nuclei in rubricytes (arrows). Wright-Giemsa stain; bar = 20 μm.

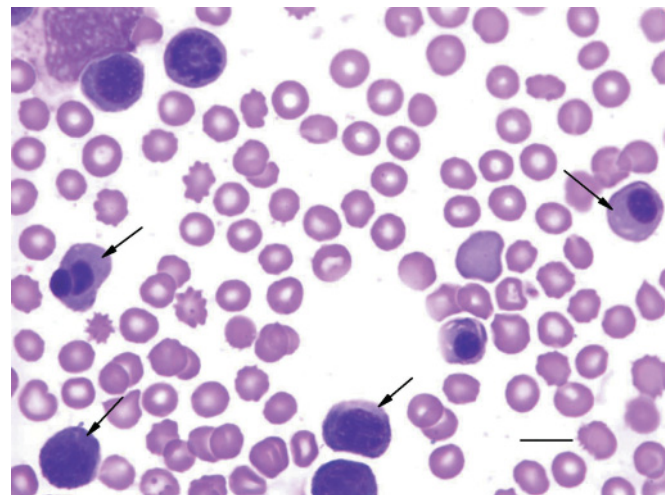


FIGURE 66.2 Mild megaloblastic dyserythropoiesis in a bone marrow aspirate from a dog with refractory anemia. Note the large size of nuclei in rubricytes relative to their maturity (arrows). Wright-Giemsa stain; bar = 10 μm.

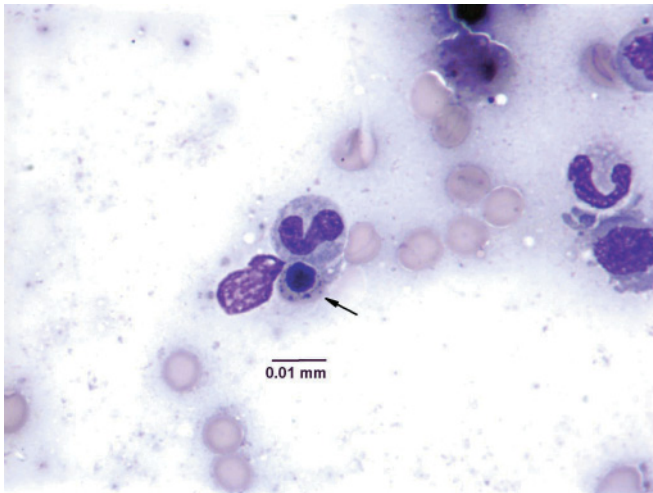


FIGURE 66.3 Ringed sideroblast (arrow) in a bone marrow aspirate from a dog with sideroblastic-type refractory cytopenias with multilineage dysplasia. Wright-Giemsa stain.

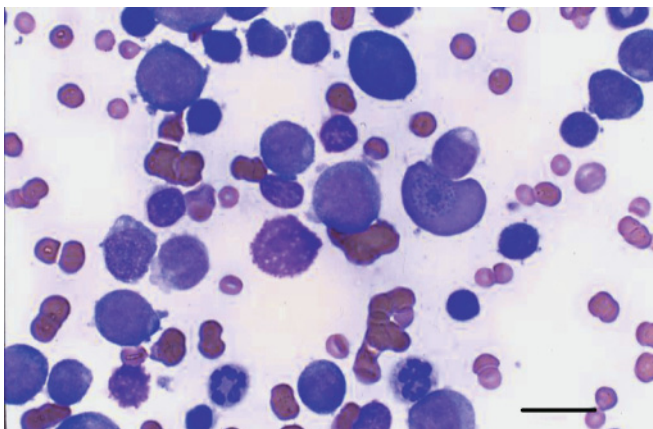


FIGURE 66.4 Increased numbers of myeloblasts and multilineage dysplasia in a bone marrow aspirate from a cat with refractory anemia with excess blasts. Note a large megaloblastic rubricyte and hypersegmented neutrophils. Wright-Giemsa stain; bar = 20µm.

anemia with excess blasts-I is defined as 5–10% myeloblasts in bone marrow and RAEB-II is defined as 10–20% myeloblasts in bone marrow (Fig. 66.4).¹³ In humans, MDS associated with isolated del(5q) chromosome abnormality has been shown to be sufficiently homogeneous to merit its own categorical classification. Unclassified forms of MDS include hypoplastic MDS and MDS with fibrosis. Acute myeloid leukemia is defined as greater than 20% myeloblasts in bone marrow. Chronic myelomonocytic leukemia is not included in the definition of MDS.

In addition to the WHO classification system, an International Prognostic Scoring System has been developed.³ This system combines the percentage of myeloblasts, karyotype, and the number of cytopenias to generate a score that estimates both survival and risk of transformation to acute leukemia.³ This scoring system can be combined with morphologic criteria to provide even more accurate prognostic information.³

A modified classification system has been described for dogs and cats.^{20,23,32} In this system, major categories include MDS with refractory cytopenias, MDS with sideroblastic differentiation, and MDS with excess myeloblasts. Myelodysplastic syndrome with refractory cytopenia is characterized by nonregenerative anemia or multiple cytopenias in the blood, dysplastic features usually most prominent in the erythroid lineage, and less than 5% myeloblasts in bone marrow. Myelodysplastic syndrome with sideroblastic differentiation is characterized by microcytic or hypochromic anemia and sideroblasts and ringed sideroblasts in bone marrow.³⁹

MYELODYSPLASTIC SYNDROMES IN DOGS

In a retrospective study of clinical bone marrow reports at a veterinary teaching hospital, MDS was reported in 4% of dogs, whereas acute leukemia of all types was observed in 6.4% of dogs.^{36,41} If classified by the WHO system, 27% would be classified as RA, 62% as RAEB, and 11% as RCMD (Table 66.2).⁴¹

TABLE 66.2 Classification and Characteristics of Myelodysplastic Syndromes in Dogs

Condition	Signalment	Clinical Signs	Peripheral Blood Findings	Bone Marrow Findings
RA	Middle-aged to old	Lethargy, exercise intolerance	Normocytic normochromic nonregenerative anemia	Dyserythropoiesis, variable increase in rubriblasts, <5% myeloblasts
RARS	Middle-aged to old	Lethargy, exercise intolerance	Nonregenerative anemia ± thrombocytopenia or leukopenia	Dyserythropoiesis, variable increase in rubriblasts, sideroblasts, ringed sideroblasts
RCMD	Middle-aged to old	Uncertain	Normocytic normochromic nonregenerative anemia	Dysplasia in multiple cell lineages, <5% myeloblasts
RAEB	Middle-aged to old	Lethargy, anorexia, fever, petechial hemorrhage	Bicytopenia or pancytopenia	Dyserythropoiesis, dysgranulopoiesis, dysmegakaryopoiesis, 5–20% myeloblasts

Refractory Anemia

Dogs with RA (also called MDS with refractory cytopenias) are typically greater than 7 years old with no breed predilection (Table 66.2).^{21,40,41} Affected dogs have an insidious onset of clinical signs consisting of lethargy and exercise intolerance. Pale mucous membranes, increased heart and respiratory rates, and anemia-related heart murmur are frequent findings on physical examination. Hematologic alterations consist of moderate to severe nonregenerative normocytic normochromic anemia. Total leukocyte and platelet counts are usually within reference ranges. Bone marrow is normocellular or hypercellular with a normal or decreased myeloid-to-erythroid ratio. Rubriblasts are frequently increased in number ranging between 5% and 30% of all nucleated bone marrow cells and myeloblasts are consistently less than 5% of all nucleated bone marrow cells.⁴¹ Dysplastic features are usually limited to the erythroid series. Features of dyserythropoiesis include binucleation, megaloblastosis, nuclear fragmentation, and asynchronous maturation.^{38,41}

Treatment of dogs with RA has included cyclophosphamide, cytosine arabinoside, and erythropoietin.^{7,30,41} Most dogs for which follow-up was available survived for months to years with no reports of progression to acute leukemia.^{7,41} Of three dogs treated with recombinant erythropoietin, all had significant improvement in hematocrit and were nonanemic at last contact.^{7,41} In the limited number of cases reported, response to treatment or survival did not appear to be dependent on the percentage of rubriblasts in bone marrow.

Refractory Anemia with Ringed Sideroblasts

Several cases of MDS with sideroblastic differentiation have been described.^{36,39,41} However, because these conditions consistently had prominent dysplastic features in all cell lineages, they are best categorized as RCMD.

Refractory Cytopenia with Multilineage Dysplasia

Refractory cytopenia with multilineage dysplasia is characterized by prominent dysplastic features in two or more hematopoietic cell lineages but no increase in myeloblasts in bone marrow.^{11,33,36,39,41} Dogs with RCMD include dogs with sideroblastic- and nonsideroblastic-types of anemia (Table 66.2).^{21,36,39} Several dogs with nonsideroblastic anemia had normocytic normochromic nonregenerative anemia, thrombocytopenia, and dysplastic features in all hematopoietic cell lineages.^{11,21} One dog was treated with low-dose aclarubicin, a differentiation-induction therapy. Hematologic improvement resulted and the dog survived for more than 2 years. Another dog was treated with prednisolone, cyclosporine, and hydroxycarbamide but died of congestive heart failure. Another dog was maintained for over 2 years with treatment consisting of prednisolone and cytarabine ocfosfate.¹⁸

Dogs with sideroblastic-type RCMD tended to have an insidious onset of clinical signs consisting of lethargy

and exercise intolerance. Pale mucous membranes and increased heart and respiratory rates are frequent findings on physical examination. Hematologic alterations consist of a microcytic or hypochromic nonregenerative anemia with many siderocytes in blood smears. The presence of siderocytes can be confirmed by staining the iron-containing granules with Prussian blue stain. Bone marrow is normocellular or hypercellular and usually contains increased amounts of hemosiderin. A characteristic feature is the presence of five or more large iron deposits in rubricytes and metarubricytes.³⁹ Many rubricytes and metarubricytes in normal adult dogs contain 1–3 small siderotic granules that are visible when stained with Prussian blue stain.⁹ Variable numbers of ringed sideroblasts are also present. Although rubriblasts are frequently increased, myeloblasts are usually less than 5% of all nucleated cells. Too few cases of sideroblastic-type RCMD have been reported to establish treatment protocols or to evaluate outcome. However, among the few cases observed by the author, both response to treatment and survival time appear to be less than that for RA. Cases of RCMD must be differentiated from sideroblastic anemia. Sideroblastic anemia has been associated with inflammatory diseases, myelofibrosis, and chloramphenicol therapy.^{15,33}

Refractory Anemia with Excess Blasts

Refractory anemia with excess blasts (also termed MDS with excess blasts) is the most frequently occurring primary type of myelodysplastic syndrome in dogs.^{21,40,41} RAEB is a disease of middle-aged to old dogs (range 4–13 years) with no apparent breed or sex predilection (Table 66.2).⁴¹ Dogs with RAEB usually appear more ill than those with RA. Signs of illness include lethargy, depression, anorexia, diarrhea, and fever.⁴¹ These signs may appear acutely or progressively worsen over weeks to months. Hematologic alterations include moderate to severe normocytic normochromic nonregenerative anemia, neutropenia, and thrombocytopenia. Bone marrow is usually hypercellular with an increased myeloid-to-erythroid ratio. A hallmark feature of RAEB is the presence of increased numbers of myeloblasts that vary from 6% to 20% of all nucleated cells in bone marrow.⁴¹ Dysplastic features are typically present in all bone marrow cell lineages. Features of dysgranulopoiesis include small myeloblasts and promyelocytes, giant metamyelocytes and band cells, asynchronous maturation, and hypersegmentation.^{3,38} Features of dyserythropoiesis include megaloblastic changes, asynchronous maturation, binucleation or trinucleation, nuclear fragmentation, and micrometarubricytes.^{3,38} Features of dysmegakaryopoiesis include asynchronous maturation, large hypolobulated forms, dwarf megakaryocytes, and dispersed nuclei.^{3,38}

Treatment of RAEB in dogs has been mostly supportive.^{11,21,41} Broad spectrum antibiotics are indicated when severe neutropenia, fever, or sepsis is present. Red blood cell (RBC) or whole blood transfusions are indicated to treat severe anemia. Erythropoietin and

other hematopoietic growth factors have been given. Although these treatments may improve the cytopenias, they do not appear to significantly prolong survival. Administration of chemotherapeutic agents, including hydroxyurea, low-dose cytosine arabinoside, and low-dose aclarubicin has been tried in individual cases. Hydroxyurea has been used most frequently. Some individual animals appear to respond to chemotherapy. At present, survival times of dogs with RAEB are short, ranging from days to months.^{11,41} Death or euthanasia is frequently associated with sepsis, organ failure, bleeding, or disseminated intravascular coagulopathy. Progression to acute myeloid leukemia has been documented in approximately 25% of reported cases. In general, survival and progression to acute myeloid leukemia can be predicted by the percentage of myeloblasts present in bone marrow.

Myelodysplastic Syndrome Unclassifiable

Variant forms of MDS are occasionally seen. These probably result from different combinations of genetic mutations in hematopoietic stem cells. A primary dysmegakaryopoiesis is one variant form.³¹ Affected dogs have a history of chronic moderate to severe thrombocytopenia that is unresponsive to immunosuppressive therapy. In bone marrow, dysplastic features, including large hypolobulated forms, dwarf megakaryocytes, asynchronous maturation, and discrete rather than lobulated nuclei, are limited to megakaryocytes. Causes of secondary dysmegakaryopoiesis, including immune-mediated hemolytic anemia, immune-mediated thrombocytopenia, ehrlichiosis, and lymphosarcoma, should be ruled out before a diagnosis of MDS is made.³¹ Affected dogs appear to have prolonged survival.

Secondary Myelodysplastic Syndromes

Secondary MDS is a clonal disorder of hematopoietic stem cells that is associated with drug or radiation treatment or toxin exposure.²⁶ In humans, secondary MDS has been associated with benzene exposure and treatment with alkylating agents and radiation. Secondary MDS frequently occurs months to years after exposure. Drug-associated MDS has not been reported in dogs but dysmegakaryopoiesis has been reported to occur as a preleukemic change in dogs after exposure to gamma radiation.²⁷ With ever increasing use of chemotherapeutic agents and radiation therapy in veterinary medicine, secondary MDS may be recognized more frequently.

SECONDARY DYSMEYELOPOIESIS IN DOGS

Diseases/Drugs/Toxins

Secondary dysmyelopoiesis includes nonclonal dysplastic conditions that should not be classified as MDS. Secondary dysmyelopoiesis is associated with a variety of conditions including specific disease processes, administration of certain drugs (see Chapter 16), expo-

sure to heavy metals (see Chapter 17) iron deficiency (see Chapter 26), and some congenital conditions (see Chapter 30).^{14,31,33,38} In one study, the most frequent diseases associated with secondary dysmyelopoiesis in dogs included immune-mediated hemolytic anemia/immune-mediated thrombocytopenia (45%), lymphosarcoma (25%), and myelofibrosis (15%).³⁸

A variety of drugs have been associated with dysmyelopoiesis. These include chemotherapeutic drugs, phenobarbital, estrogen, cephalosporins, chloramphenicol, and colchicine.^{1,15,19} Drugs that interfere with DNA synthesis, including antimetabolites (e.g. azathioprine, cytosine arabinoside, hydroxyurea), folate antagonists (e.g. methotrexate), plant alkaloids (e.g. vincristine), and alkylating agents (e.g. cyclophosphamide) frequently induce dysplastic changes.^{1,5} These dysplastic changes are usually most prominent in the erythroid lineage.^{5,32} Binucleation, fragmented nuclei, giant forms, atypical mitotic figures, and megaloblasts are frequently observed. Phenobarbital therapy has been associated with a severe normocytic normochromic nonregenerative anemia and dyserythropoiesis.¹⁹ In general, the dysplastic features are identified during treatment and resolve within days after cessation of treatment. This is unlike drug- or toxin-induced secondary MDS in which dysplastic features are identified months to years after exposure and persist. Sideroblastic anemia was documented in a dog treated with chloramphenicol.¹⁵

Secondary dysmyelopoiesis has also been associated with iron deficiency, lead, zinc, and arsenic poisoning, and with leishmaniasis.^{5,10,12,14,15,29} Iron deficiency is characterized by a microcytic, hypochromic anemia. Dysplastic features are restricted to the erythroid lineage and are characterized by rubricytes and metarubricytes with a ragged rim of cytoplasm and patchy areas of basophilia within the cytoplasm.¹⁴ A late stage maturation arrest in the erythroid series may be present in some dogs. Lead inhibits several enzymes in the heme synthesis pathway.¹² Resultant inhibition of heme synthesis leads to iron accumulation in erythroid precursors and sideroblastic anemia. Lead also inhibits a pyridine specific 5' nucleotidase that is involved in degradation of ribosomal ribonucleic acids. Retention of ribosomes may result in basophilic stippling. Arsenic poisoning causes marked dyserythropoiesis characterized by karyorrhexis, megaloblastosis, and multinuclearity. Leishmania infection in dogs has been associated with dysmegakaryopoiesis and dyserythropoiesis.¹⁰

Variant forms of secondary dysmyelopoiesis include selective dysmegakaryopoiesis and sideroblastic anemia.^{31,33} Dysmegakaryopoiesis is associated with the same spectrum of disease conditions as other forms of secondary dysmyelopoiesis. Secondary dysmegakaryopoiesis must be differentiated from megakaryoblastic leukemia. Little or no increase in megakaryoblasts is seen in secondary dysmegakaryopoiesis, whereas, megakaryoblastic leukemia is characterized by a moderate to marked increase in megakaryoblasts in blood or bone marrow.³¹ Unlike other types of secondary dysmyelopoiesis, sideroblastic anemias are associated with inflammatory diseases and myelofibrosis.³³ Affected

TABLE 66.3 Classification and Characteristics of Myelodysplastic Syndromes in Cats

Condition	Signalment	Clinical Signs	Peripheral Blood Findings	Bone Marrow Findings
RA	Reported cases presently classified as RCMD			
RARS	Few cases reported			
RCMD	Variable	Anorexia, depression, weight loss, weakness	Macrocytic normochromic nonregenerative anemia, bicytopenia or pancytopenia, metarubricytosis	Dysplasia in multiple cell lineages, <5% myeloblasts
RAEB	Variable	Lethargy, anorexia, fever, petechial hemorrhage	Normocytic or macrocytic nonregenerative anemia, bicytopenia or pancytopenia	Dyserythropoiesis, dysgranulopoiesis, dysmegakaryopoiesis, 5–20% myeloblasts

dogs have a moderate to severe microcytic or hypochromic anemia that may be confused with iron deficiency anemia. Dysplastic features are similar to those associated with RARS except that the percentage of rubriblasts in bone marrow is not increased. Dysplastic features are restricted to or are most prominent in the erythroid lineage. The role of pyridoxine deficiency as a cause of the sideroblastic changes has not been investigated.

Differentiating MDS from secondary dysmyelopoiesis is challenging.^{5,32} Bone marrow features that differentiate MDS from secondary dysmyelopoiesis have been proposed.⁵ This author proposed that the diagnosis of MDS be restricted to dogs with dysplastic features in more than one cell lineage and that have greater than 5% myeloblasts in bone marrow. With these criteria, all cases of RA would be categorized as secondary dysmyelopoiesis despite being classified as MDS in the human classification system. Other authors prefer a less restrictive definition.^{23,32} In general, in secondary dysmyelopoiesis, the percentage of dysplastic cells is lower and the percentages of rubriblasts and myeloblasts are not increased. Exceptions include sepsis and bone marrow recovering from toxic injury. In the case of sepsis, a combination of increased myelopoiesis and rapid mobilization of mature myeloid cells into the blood results in a shift toward immaturity in the myeloid series. In marrow recovering from suppression, a wave of immature cells may be seen, resulting in a high percentage of blasts cells.²⁴ The presence of toxic changes in the myeloid cells is helpful in identifying sepsis-associated dysmyelopoiesis. Dysplastic features are usually more numerous in MDS compared to secondary dysmyelopoiesis and megaloblastic features are usually more prominent, but in individual cases these differences may be insufficient to make a conclusive diagnosis. Therefore, diagnosis necessitates evaluation for diseases, drug treatments, and toxin exposures that are known to cause secondary dysmyelopoiesis.³⁸

MYELODYSPLASTIC SYNDROMES IN CATS

In a retrospective study of clinical bone marrow reports at a veterinary teaching hospital, 11.6% had a diagnosis

of MDS. Of these, 4.3% had a diagnosis of RA, 0.5% had a diagnosis of RCMD, and 6.8% had a diagnosis of RAEB (Table 66.3).³⁷ A clonal origin of MDS in FeLV-infected cats has been documented.¹⁶ Cytopenia in cats with MDS has been associated with decreased numbers of granulocytes-monocyte colony-forming units and erythroid colony-forming units in four of seven cats tested and decreased size of granulocytes-monocyte colony-forming units in all seven cats tested.²² The probability of progression of FeLV-positive MDS cats to acute myeloid leukemia is considered to be high.⁶

Refractory Anemia

Cats previously categorized as RA have multiple cytopenias in the blood and dysplastic features in two or more cell lineages in blood or bone marrow. Therefore, they should be categorized as RCMD.³⁵

Refractory Anemia with Ringed Sideroblasts

Several cats with sideroblastic anemia have been described.^{17,25,35} At least one of these cats fits into the category of RARS.³⁵ Unlike dogs, cats with RARS have a macrocytic normochromic anemia. They also have dyserythropoiesis, less than 5% myeloblasts in bone marrow, the presence of siderocytes in blood and bone marrow and variable numbers of ringed sideroblasts. Healthy cats do not have siderocytes in bone marrow.⁵ Increased eosinophil and basophil precursors were observed in bone marrow in one cat.³⁵ Eosinophilia in bone marrow is a frequent finding in cats with MDS.^{20,25}

Refractory Cytopenia with Multilineage Dysplasia

Cats with previous diagnoses of RA and MDS-RC and some cats with a diagnosis of RARS appear to fit in this category because of the presence of multiple cytopenias in the blood and multilineage dysplasia in bone marrow (Table 66.3).^{17,25,35} A few cats with macrocytic normochromic anemia, siderocytes, ringed sideroblasts, and multilineage dysplasia have been reported.^{17,25} The age and FeLV status of these cats varied between studies. In some studies, the median age was approximately 3

years old and 70–90% of the cats were FeLV positive.^{5,17,25} In another study, only 38% of cats were FeLV positive and the median age was 8.7 years old.³⁵ In general, these cats were less severely ill than cats with RAEB. Frequently encountered clinical signs include anorexia, depression, weakness, and loss of body mass. Affected cats have a moderate to severe macrocytic normochromic nonregenerative anemia and frequently have metarubricytosis and autoagglutination. Most cats are thrombocytopenic and some are leukopenic. Bone marrow is normocellular or hypercellular, myeloblasts are <5% of all nucleated cells and rubriblasts are typically increased. Dysplastic features are present in the erythroid line of all cats and frequently present in granulocyte and megakaryocyte lineages. Features of dysgranulopoiesis include maturation arrest, hypersegmentation, hyposegmentation, giant band and segmented neutrophils, and asynchronous maturation of nucleus and cytoplasm. Features of dyserythropoiesis included asynchronous maturation of nucleus and cytoplasm, binucleation, fragmented or lobulated nuclei, and megaloblastosis. Features of dysmegakaryopoiesis included large megakaryocytes with mature-appearing granulated cytoplasm but poorly lobulated or multiple discrete nuclei, megakaryocytes with mature hyperlobulated nuclei but immature-appearing homogeneous blue cytoplasm, and many small megakaryocytes with single or multiple discrete round nuclei. Evaluation of bone marrow core biopsy specimens revealed myelonecrosis and myelofibrosis in one-third of cats with RCMD.³⁵

Although prognosis is guarded, some cats with RCMD respond partially or completely to symptomatic therapy and have prolonged survival.³⁵ Refractory anemia and RCMD appear to have less tendency to progress to acute myeloid leukemia and have a longer survival times compared to RAEB. However, this difference is difficult to prove statistically when small groups are compared.¹⁷ Therapy has consisted of prednisolone, prednisolone and cytarabine, cyclosporine A, or combination chemotherapy consisting of daunorubicin, cytarabine, vincristine, and prednisolone.¹⁷

Refractory Anemia with Excess Blasts

Cats with RAEB are characterized by multiple cytopenias, multilineage dysplasia, and increased myeloblasts in bone marrow (Table 66.3). In studies in which most affected cats were FeLV-positive, the median age was approximately 3 years, whereas, in studies in which the majority of affected cats were FeLV-negative, the median age was approximately 9 years.^{17,25,35} Cats with RAEB tended to be severely ill with clinical signs including anorexia, weakness, depression, loss of body mass, fever, vomiting, diarrhea, and platelet-related bleeding. Affected cats have a moderate to severe nonregenerative anemia and some have metarubricytosis and autoagglutination. Most cats are thrombocytopenic and some are leukopenic. Bone marrow is usually normocellular or hypercellular but may be hypocellular. Erythroid hypoplasia is a relatively frequent finding in FeLV-positive cats. All cats had 6% to 20% myeloblasts

in bone marrow with variable increases in rubriblasts. Dysplastic features are present in all cell lines. Features of dysgranulopoiesis, dyserythropoiesis, and dysmegakaryopoiesis are similar to those associated with RCMD. Myelonecrosis and myelofibrosis are present in bone marrow of some affected cats.³⁵ Presently, survival for cats with RAEB varies from days to a few months with frequent progression to acute myeloid leukemia.^{17,35}

SECONDARY DYSMELOPOIESIS IN CATS

Diseases/Drugs/Toxins

In a retrospective study of clinical bone marrow reports in cats, 6.3% were categorized as secondary dysmyelopoiesis.³⁵ Disease conditions associated with secondary dysmyelopoiesis include immune-mediated hemolytic anemia, immune-mediated thrombocytopenia, pure red cell aplasia, lymphosarcoma, glomerulonephritis, and feline infectious peritonitis.³⁵ Bone marrow has normal or increased cellularity and dysplastic features are most prominent in the erythroid series but can be found in granulocytes and megakaryocytes. In general, these changes are similar to those seen in MDS except that blast cells are not increased, the percentage of dysplastic changes is usually not as high, and megaloblasts are infrequently seen.

Myelodysplastic syndromes are difficult to differentiate from secondary dysmyelopoiesis associated with immune-mediated hemolytic anemia. Both syndromes are characterized by severe nonregenerative anemia and metarubricytosis.³⁵ Additionally, both syndromes can have autoagglutination present. Several factors are useful in differentiating these conditions. Marked macrocytosis (i.e. MCV > 65 fL) is a useful indicator of RA and RCMD. Additionally, >10% dysplastic changes in bone marrow are more consistent with MDS. Additionally, megaloblastic changes are prominent in MDS but rare in secondary dysmyelopoiesis. Additionally, cats with MDS typically have increased rubriblasts or myeloblasts while cats with secondary dysmyelopoiesis do not. An increased number of small lymphocytes in bone marrow is a consistent finding in IMHA and PRCA.³⁴

MYELODYSPLASTIC SYNDROME IN HORSES

Myelodysplastic syndrome has been described in a Quarter Horse.⁸ The MDS was characterized by a macrocytic nonregenerative anemia, leukopenia, and thrombocytopenia in the blood and a bone marrow that contained 9% myeloblasts, marked dyserythropoiesis, and increased plasma cells and eosinophils. Dyserythropoiesis was characterized by megaloblastic changes. This case appears to be most consistent with a diagnosis of RAEB.

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Acute Myeloid Leukemia

LAURA A. SNYDER

Overview of Acute Myeloid Leukemia

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Acute myeloblastic leukemia

Acute promyelocytic leukemia

Acute myelomonocytic leukemia

Acute monocytic leukemia

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Acronyms and Abbreviations

ACH_E, acetylcholinesterase; ALL, acute lymphoblastic leukemia; ALSG, Animal Leukemia Study Group; ALSV, avian leukosis/sarcoma virus; AML, acute myeloid leukemia; AML-M0, acute myeloblastic leukemia, minimally differentiated; AML-M1, acute myeloblastic leukemia without maturation; AML-M2, acute myeloblastic leukemia with maturation; AML-M3, acute promyelocytic leukemia; AML-M4, acute myelomonocytic leukemia; AML-M5a, acute monoblastic leukemia; AML-M5b, acute monocytic leukemia; AML-M6a, acute erythroleukemia; AML-M6b, acute erythroid leukemia with erythroid predominance; AML-M7, acute megakaryoblastic leukemia; AML-NOC, acute myeloid leukemia-not otherwise categorized; ANAE, alpha naphthylacetate esterase; ANC, all nucleated cell; APL, acute promyelocytic leukemia gene; ATRA, alltrans-retinoic acid; AUL, acute undifferentiated leukemia; CAE, chloroacetate esterase; CML, chronic myeloid leukemia; FAB, French-American-British; FeLV, feline leukemia virus; LAP, leukocyte alkaline phosphatase; MDS, myelodysplastic syndrome; MPD, myeloproliferative disease; MPO, myeloperoxidase; NCI, National Cancer Institute; NEC, non-erythroid cell; NSE, nonspecific esterase; PAS, periodic acid Schiffs; RARA, retinoic acid receptor-alpha; SBB, Sudan Black; WBC, white blood cell; WHO, World Health Organization.

OVERVIEW OF ACUTE MYELOID LEUKEMIA

Definition and Origin of Disease

Acute myeloid leukemias (AMLs) are a diverse group of non-lymphoid hematologic malignancies that are derived from hematopoietic stem cells. Acute myeloid leukemias are typically classified as originating from one or more of the four myeloid cell lines: granulocytic, monocytic, erythroid and megakaryocytic. Acute myeloid leukemia must be distinguished from other clonal myeloproliferative diseases (MPDs) such as chronic myeloid leukemia (CML; see Chapter 65) and

myelodysplastic syndromes (MDSs; see Chapter 66).⁸ In part, diagnosis is achieved through the identification of $\geq 20\%$ blast cells in the patient's bone marrow and/or blood.^{2,22,39} Proliferation of these blast cells in patients with AML eventually results in myelophthisis and consequently hematologic manifestation of disease.

In both humans and animals, AML may develop secondary to environmental carcinogens, immunomodulatory drugs, and prior cancer therapies, particularly topoisomerase inhibitors, alkylating agents, and ionizing radiation.^{2,3} Alternatively, AML may develop de novo with no historical exposure, or may evolve from pre-existing MDS.^{2,3,9,24,39}

Clinical and Laboratory Findings

Animals diagnosed with AML generally show non-specific clinical signs including lethargy, weakness, anorexia, pyrexia and hepatosplenomegaly. Lymphadenopathy of varying severity may or may not be present. Unique clinical features noted in the horse include, edema of the limbs and ventral abdomen, harsh lung sounds, epistaxis and ulceration of the skin and mucous membranes.^{11,25,30,31,33} Less commonly, severe systemic infections, such as aspergillosis, may occur.^{4,9}

Common hematologic changes include moderate to severe nonregenerative anemia and thrombocytopenia. Changes in the leukogram are somewhat more variable. In overt cases of leukemia, a marked leukocytosis is often present with numerous circulating blast cells identified. These cases may be complicated by the development of leukostasis, where large numbers of circulating cells sludge within the vasculature of vital organs, particularly the lungs and brain.⁵ Occasionally, few or no identifiable blast cells are noted in circulation. In such cases the terms subleukemic leukemia or aleukemic leukemia are applied, and diagnosis hinges on the identification of $\geq 20\%$ blast cells in bone marrow.

In some cases of AML, light microscopic differentiation from acute lymphoblastic leukemia (ALL) or stage V lymphoma with circulating blast cells may prove challenging if not impossible. When compared to lymphoblasts, blast cells of AML are classically larger in size, have a lower nuclear to cytoplasmic ratio, variable nuclear contours, a more delicate chromatin pattern, more abundant cytoplasm and may contain cytoplasmic vacuoles or few to many fine azurophilic granules.^{1,13,23,37} However in some cases of AML, light microscopic differentiation from acute lymphoblastic leukemia (ALL) or stage V lymphoma with circulating blast cells may be impossible. In these instances, cytochemical staining and immunophenotyping are essential tools for definitive diagnosis of AML.

Grossly and histologically, organ involvement in AML may exhibit patterns distinct from those observed in some lymphomas. Splenic enlargement is typically symmetrical and diffuse. Histologically, there may be diffuse filling of the sinuses with neoplastic cells, loss of germinal center architecture and atrophy of periarteriolar lymphoid sheaths. Hepatic involvement is variable and may consist of sinus infiltration with neoplastic cells, centrilobular ischemic degeneration and focal hemorrhagic infarction. Lymph nodes typically exhibit variable degrees of lymphoid atrophy and infiltration of the medullary cords. As pthysis of the marrow progresses, lymph nodes and other organs may be colonized by benign bone marrow precursors in an effort to establish extramedullary hematopoiesis. Care must be taken not to rely solely on patterns of organ involvement, as both AML and ALL can appear similar.³⁸ Ultimately a multifaceted approach utilizing cytochemical stains, immunophenotyping, histology, and cytogenetic analysis (if available) will provide the most complete and accurate diagnosis.^{14,26,27,32,34,41}

COMPARATIVE REVIEW OF THE CLASSIFICATION SCHEME OF MYELOID NEOPLASMS

French-American-British Classification

The French-American-British (FAB) cooperative group first proposed a classification scheme for the diagnosis of leukemia in 1976 (see Chapter 64). The goal of this meeting was to develop a system of classification that would allow for inter-case and inter-hospital comparisons and case prognostication. The proposals generated six sub-classifications of AML (M1–M6) based on a 30% blast count in bone marrow or blood and morphologic and cytochemical findings.³ Revisions to the criteria for classification of AML were subsequently published in 1985 in order to address imprecision in the original scheme with regard to differentiation of these sub-classifications.^{2,13,24,40}

The FAB classification scheme provided a consistent morphological and cytochemical framework for the diagnosis and classification of AML. This framework allowed for identification of specific genetic defects associated with leukemic states. In some instances the morphology of AML classification was able to reliably predict the genetic abnormality. But unfortunately, in many cases, correlations were not identified or were incorrect. These findings led to the realization that there was more genetic diversity among leukemic states than could be predicted by morphology and cytochemistry alone and prompted subsequent revisions by the World Health Organization (WHO).^{2,40}

World Health Organization Revisions

In 2001, the WHO proposed revisions to the FAB classification based on concepts derived from clinical and scientific studies (Table 67.1). This reclassification is based on utilization of not only morphologic characteristics, but also incorporation of genetic aberrations, immunophenotypes, and biologic and clinical findings. Combined, these are a better predictor of prognosis than morphology alone.^{2,40}

Classification is modified to reflect two main subgroups with differing pathogeneses: AML that develops de novo versus AML that occurs secondary to MDS or cancer therapy. In general, de novo AML is more responsive to chemotherapy, has no association with dysplastic morphologies, is associated with a single, low-risk cytogenetic defect, and has no age predisposition. In contrast, AML that develops secondary to MDS or chemotherapy is generally associated with a poorer prognosis, has multiple, high-risk cytogenetic defects, and is typically diagnosed in older patients.^{2,10,14,22,34,40} Current categories of AML in lieu of AML-M numerical classification scheme are as follows: AML with recurrent genetic abnormalities, AML with multi-lineage dysplasia (with or without prior MDS), therapy-related AML and AML not otherwise categorized (AML-NOC). AML with recurrent genetic abnormalities includes four well-defined genetic abnormalities that are

TABLE 67.1 WHO Classification of Acute Myeloid Leukemia

Acute myeloid leukemia with recurrent genetic abnormalities
AML with t(8;21)(q22;q22)
AML with abnormal bone marrow eosinophils and inv(16) (p13;q22) or t(16;16)(p13q22)
Acute promyelocytic leukemia with t(15;17)(q22;q12)
AML with 11q23 abnormalities
Acute myeloid leukemia with multilineage dysplasia
With prior MDS or MPD
Without prior MDS or MPD but with dysplasia in $\geq 50\%$ of cells in 2 or more myeloid lineages
AML and MDS; therapy related
Prior alkylating agent or radiation
Prior topoisomerase inhibitor
AML-NOC (not otherwise categorized)
Acute myeloid leukemia, minimally differentiated (AML-M0)
Acute myeloid leukemia without maturation (AML-M1)
Acute myeloid leukemia with differentiation (AML-M2)
Acute myelomonocytic leukemia (AML-M4)
Acute monocytic/blastic leukemia (AML-M5a/b)
Acute erythroid/erythroid predominant leukemia (AML-M6a/b)
Acute megakaryoblastic leukemia (AML-M7)
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma

typically associated with de novo AML. Most genetic abnormalities in this group are balanced translocations and inversions. Specifically they are t(8;21)(q22;q22), inv(16)(p13q22), t(15;17)(q22;q12), and abnormalities of 11q23. As additional genetic defects and their behaviors are identified, this sub-category of AML will likely expand, and consequently the category of AML-NOC will likely become smaller.^{2,10,14,22,34,40}

Another notable change implemented in the WHO classification guidelines is lowering of the blast threshold from 30% to 20%. This recommendation is based on observations of the biologic behavior of MDS with blast cells in the range 20–29%. In the majority of cases, the disease process followed a similar, aggressive course to that of AML with multi-lineage dysplasia. This has led to the suggestion that the MDS with excess blasts in transformation is actually the same disease process as AML.^{2,10,22,40}

Animal Leukemia Study Group Adaptations

In 1991 the Animal Leukemia Study Group (ALSG) sought to determine the applicability of the FAB classification scheme to veterinary patients. Veterinary hematopathologists utilized bone marrow and blood smears from 49 dogs believed to have MPD to adapt the human classification scheme. Subsequently, the 30% marrow or blood blast threshold and phenotypic AML-M classification scheme was adopted along with modifications of certain criteria and the addition of other parameters.^{17,23}

Blast cells were redefined to include only type I and II myeloblasts, monoblasts and megakaryoblasts in AML-M1 through M5 and M7. Type III and IV myelo-

blasts, abnormal promyelocytes and promonocytes were either not recognized or specifically excluded. Because of the high prevalence of disease in feline leukemia virus (FeLV)-positive cats, AML with an erythroid phenotype (AML-M6) was expanded to include features not originally specified in the FAB scheme. Specifically, blast cells for AML-M6 were to be enumerated out of all non-erythroid cells (NECs), rather than all nucleated cells (ANCs).^{17,23} Furthermore, AML-M6 was redefined to include a second phenotype, AML-M6Er, in which there is minimal myeloid or monocytoid contribution to the blast cell proliferation. This subtype, also referred to as pure erythroleukemia or erythroleukemia with erythroid predominance, was defined to have $\geq 30\%$ of ANC as blasts *and* to include rubriblasts, in addition to myeloblasts, in this count. Similar modifications were eventually also introduced into the human classification scheme.^{2,17,23,40}

Final additions and revisions included the recognition of acute undifferentiated leukemia (AUL) as a specific entity and recommendations not to separate AML of monocytoid origin into AML-5a (monoblastic) and AML-5b (monocytic) due to poor agreement.^{3,17,21,39,23} Acute promyelocytic leukemia (AML-M3) was not recognized in the ALSG case set and criteria for its diagnosis were not included in their recommendations.^{3,17,23} To date, spontaneous occurrence AML-M3 has only been recognized in a single pig.⁷ Though acute megakaryoblastic leukemia (AML-M7) was not identified in the ALSG case set, criteria for its diagnosis were still included and described in their recommendations.^{17,36}

At this time, the veterinary pathology community continues to utilize the AML-M classification system; however, reclassification, as supported by the recent identification of recurrent chromosomal abnormalities in canine leukemia and lymphoma that are similar or identical to those found in the homologous human diseases,⁹ will likely be apparent as cytogenetic studies become practical in veterinary patients. Currently, there is no consensus regarding the use of 20% versus 30% blast cells in the bone marrow and/or blood as a cutoff for diagnosis of AML. The impetus for this change in human medicine was that the 20% cutoff better predicted biological or clinical features. Identification of a similar phenomenon in veterinary patients will likely be necessary before an agreement is reached. Tables in this chapter reflect a 20% blast cutoff for diagnosis of AML.

Recommendations for Diagnostic Work-up

Currently, the WHO recommendations for diagnostic evaluation of humans with suspected AML include a 200-cell differential white blood cell (WBC) count and a 500-cell differential count on a bone marrow aspirate stained using either Wright-Giemsa or May-Grünwald-Giemsa procedures. A bone marrow core biopsy also should be evaluated for architectural changes such as myelofibrosis, and for evaluation of focal hematopoietic disease. Panels of histochemical and cytochemical stains should be employed for confirmation of the origin of

blast cells. Finally, cytogenetic analysis is also an essential component of the diagnostic evaluation for AML.^{2,22,40} With the exception of genetic analysis, the preceding recommendations are readily available for characterization of AML in veterinary species.

DIAGNOSIS OF ACUTE MYELOID LEUKEMIA

Nomenclature and Terminology

The cell types that give rise to AML, as well as the cytochemical stains and the immunologic markers used to define the maturation stages in the myeloid lineages are described in Chapters 132 and 144. The terminology used to describe AMLs in the literature is inconsistent and can be confusing. The following definitions are provided to clarify communication and case comparison in both the clinical and research settings.

The proper umbrella term for acute non-lymphoid leukemias (granulocytic, monocytic, megakaryocytic and erythroid) is acute myeloid leukemia (AML), rather than acute myeloblastic leukemia. The term *myeloblastic* should be reserved for specific subtypes of leukemia (M0, M1 and M2), where immature blast forms predominate.^{2,23,40} The term *maturation* refers to morphologic changes that imply development of cellular functions. In leukemias with *maturation*, variable percentages of nucleated cells exhibit development beyond the blast stage (i.e. promyelocyte, myelocyte and metamyelocyte). These include AML-M2 through AML-M7.^{2,23,40} The term *differentiation* implies commitment to a specific lineage (i.e. granulocytic, monocytic, megakaryocytic, erythroid or lymphoid). In *undifferentiated* leukemia, cells lack ultrastructural, morphologic, and cytochemical features that allow lineage designation. Therefore, in acute undifferentiated leukemia (AUL) lymphoid versus myeloid origin cannot be determined. In leukemias with *minimal differentiation*, early lineage commitment is evident only with the use of immunophenotyping and/or electron microscopy.^{2,17,23,40} Traditionally, the term *blast* has been used to refer to the most immature or primitive cell within a certain lineage. However, the definition of a *blast* is expanded for the diagnosis of AML in human medicine. Precisely what constitutes a *blast* depends to some extent on the ultimate diagnosis. Blast counts are typically determined as a percentage of ANCs in the bone marrow with the exception of lymphocytes, plasma cells, mast cells, macrophages and cells involved in another hematopoietic neoplasm. Myeloblasts are always counted as part of the blast cell percentage in AML, with some other cells counted as blast equivalents. These equivalents include monoblasts in acute monocytic leukemia (AML-M5), monoblasts and promonocytes in acute myelomonocytic leukemia (AML-M4), abnormal promyelocytes in acute promyelocytic leukemia (AML-M3) and megakaryoblasts in acute megakaryoblastic leukemia (AML-M7). There is a slight paradigm shift for the erythroleukemias in veterinary medicine. In acute erythroleukemia (AML-M6a), blasts are counted as a

percentage of non-erythroid cells (NEC) rather than as a percentage of ANCs, and in acute erythroleukemia with erythroid predominance (AML-M6b) erythroblasts are included as part of the blast count, in addition to myeloblasts (Table 67.2).^{2,17,23,40}

Subtypes of Acute Myeloid Leukemia in Veterinary Medicine

Acute Undifferentiated Leukemia (AUL)

Acute undifferentiated leukemia represents a leukemic process in which the predominant cell is so immature it cannot be identified as being of either myeloid or lymphoid origin. Cells lack all identifiable morphologic, ultrastructural, immunologic, and cytochemical markers, thus precluding lineage assignment. Blast cells have a nondescript appearance and may have eccentric nuclei, prominent nucleoli, moderately basophilic cytoplasm, pseudopodia, and occasionally a few fine granules. Importantly, AUL blast cells are negative for traditional immunophenotypic markers with the exception of CD34, identifying them only as being of hematopoietic origin. In humans, AUL is associated with a poor prognosis.^{13,17,21,37,38,39}

Acute Myeloblastic Leukemia (AML-M0, M1, M2)

Acute myeloblastic leukemia may be divided into three subtypes: acute myeloblastic leukemia, minimally differentiated (AML-M0), acute myeloblastic leukemia without maturation (AML-M1), and acute myeloblastic leukemia with maturation (AML-M2). In all acute myeloblastic leukemias, myeloblasts comprise $\geq 20\%$ of ANCs, though blast percentages are typically much higher and may approach 100%.^{13,17,37,38}

While nonregenerative anemia and thrombocytopenia are common, WBC counts are variable and may be elevated, normal, or decreased. However, because of impaired maturation, neutrophil counts are frequently low.^{37,38}

AML-M0 is characterized by the presence of immature type I myeloblasts in the bone marrow and blood. These type I myeloblasts lack microscopically identifiable primary granules; thus this neoplasm cannot be distinguished from acute lymphocytic leukemia (ALL) by light microscopy. Furthermore, fewer than 3% of blast cells stain positively for myeloperoxidase (MPO), Sudan Black B (SBB) and/or chloroacetate esterase (CAE). Ultrastructural demonstration of MPO-positive granules is necessary for confirmation of myeloid lineage. Additionally, $>20\%$ of blasts should express surface markers consistent with myeloid lineage (CD13, CD33). A normal or mildly elevated leukocyte count is typically present in these patients.^{13,17,37,38}

AML-M1 is distinguished from AML-M0 by the presence of $>3\%$ myeloblasts staining positive for MPO, SBB and/or CAE. Fewer than 10% of bone marrow cells exhibit maturation to the promyelocyte stage and beyond. Additionally, fewer than 10% of myeloblasts

TABLE 67.2 Diagnostic Criteria for Acute Myeloid Leukemia Veterinary Medicine**Acute undifferentiated leukemia (AUL)**

- >20% (typically close to 100%) of ANC are immature blasts
- Blast cells lack all morphologic, ultrastructural, cytochemical, and immunologic markers
- May be CD34 positive, indicating lympho-hematopoietic origin

Acute myeloblastic leukemia, minimally differentiated (AML-M0)

- >20% (often higher or close to 100%) of all ANC are myeloblasts
- <3% of blasts stain positively for MPO, SBB or CAE
- >20% of blasts express one or more myeloid antigens (CD13, CD33)
- Myeloid lineage confirmed by ultrastructural demonstration of MPO positive granules
- Negative for lymphoid antigens

Acute myeloblastic leukemia without maturation (AML-M1)

- >20% (often higher or close to 100%) of ANC are myeloblasts
- >3% of blasts stain positively for MPO, SBB or CAE
- <10% of ANC exhibit granulocytic differentiation to promyelocyte and beyond

Acute myeloblastic leukemia with maturation (AML-M2)

- >20% of ANC are myeloblasts
- >3% of blasts stain positively for MPO, SBB or CAE
- >10% of ANC exhibit granulocytic differentiation to promyelocyte and beyond
- <20% of ANC exhibit monocytic differentiation
- +/- dysplasia in granulocytic lineage

Acute promyelocytic leukemia (AML-M3)

- Now classified under AML with recurrent cytogenetic abnormalities t(15;17)(q21;q22)
- >20% of ANC are myeloblasts and abnormal promyelocytes
- Strongly positive with MPO/SBB; positive with CAE
- Hyper and hypo/microgranular variants are recognized
- Hypergranular variant is associated with Auer rods
- Extremely rare in veterinary species; typically hypogranular

Acute myelomonocytic leukemia (AML-M4)

- >20% of ANC are myeloblasts, monoblasts and promonocytes
- >20% of ANC are maturing granulocyte lineage (MPO/SBB positive)
- >20% of ANC are maturing monocyte lineage (CD14 positive and NSE positive/negative with fluoride inhibition)
- M4-Eo variant in humans with evidence of eosinophilic differentiation

Acute monoblastic leukemia (AML-M5a)

- >80% of ANC are monocytic origin with at least 20% myeloblasts, monoblasts and promonocytes
- >50% of monocytic cells are monoblasts
- Strongly NSE positive/negative with fluoride inhibition, CD14 positive
- MPO/SBB negative

Acute monocytic leukemia (AML-M5b)

- >80% of ANC are monocytic origin with at least 20% myeloblasts, monoblasts and promonocytes
- >50% of monocytic cells are promonocytes
- Strongly NSE positive/negative with fluoride inhibition; CD14 positive
- MPO/SBB negative

Acute erythroleukemia (AML-M6a)

- >50% of ANC are erythroid origin
- May have left shifting and dysplasia of the erythroid lineage
- >20% of NECs are myeloblasts
- Most common type of erythroleukemia in humans

Acute erythroleukemia with erythroid predominance (AML-M6b / Pure erythroleukemia)

- >50% of ANC are erythroid origin
- May have left shifting and dysplasia of the erythroid lineage
- >20% of ANC are myeloblasts or rubriblasts
- Most common type of erythroleukemia in veterinary species

Acute megakaryoblastic leukemia (AML-M7)

- >20% of ANC are blasts
- >50% of blast cells are megakaryoblasts
- Megakaryoblasts are CD41/61 positive and stain with acetylcholinesterase, ANAE and PAS
- Marrows are often severely sclerotic and dry tap may result

exhibit primary cytoplasmic granulation (type I and some type II myeloblasts). Though this granulation allows for distinction from ALL, cytochemical and immunologic markers may still be necessary for accurate identification. A normal or mildly elevated leukocyte count is typically present in these patients.^{13,17,37,38}

AML-M2 is distinguished from AML-M1 by the presence of $\geq 10\%$ of bone marrow cells maturing to the promyelocyte stage and beyond. Primary cytoplasmic granulation is common and prominent due to the presence of many type II and III myeloblasts (though the latter were not identified or characterized by the ALSG). To distinguish AML-M2 from AMLs with a monocytic component, $< 20\%$ of ANCs in bone marrow should exhibit monocytic differentiation. Additionally, $> 3\%$ of blasts stain positively for MPO and/or SBB. Thrombocytopenia and nonregenerative anemia are typically present. A leukocytosis of up to 50,000 WBC/ μL , including type I, II, and III myeloblasts, promyelocytes, and rare myelocytes is also commonly noted.^{13,17,37,38}

Acute Promyelocytic Leukemia (AML-M3)

Acute promyelocytic leukemia (AML-M3) is characterized by the presence of atypical promyelocytes in bone marrow or blood. As in other types of AML, the blast percentage must comprise $\geq 20\%$ of ANCs; however, in AML-M3, both myeloblasts and atypical promyelocytes are included in the blast percentage. In humans, these atypical promyelocytes often outnumber the myeloblasts and have two distinct microscopic appearances: the more common hypergranular variant, and the less common micro- or hypogranular variant. The hypergranular variant typically presents with anemia, thrombocytopenia, and leukopenia due to myelophthisis. The hypogranular variant is reported to present with a marked leukocytosis of approximately 50,000/ μL , consisting predominantly of abnormal promyelocytes. Coagulopathies may be noted at the time of presentation or may develop after initiation of therapy. Diagnosis may be confirmed with positive staining for SBB, MPO and CAE.^{13,17,37,38}

In humans, AML-M3 has been reclassified as AML with a recurrent genetic abnormality as it is associated with the chromosomal translocation $t(15;17)(q22;q11-12)$. This results in the fusion of the retinoic receptor-alpha (RARA) gene with the zinc finger binding transcription factor, also known as the promyelocytic leukemia (PML) gene. This fusion product is known as PML-RARA. It results in impaired maturation and uncontrolled proliferation including down-regulation of repressor genes through limited hypermethylation, heterochromatin formation, and gene silencing. This unique pathogenesis allows for highly effective targeted therapy using all-trans-retinoic acid (ATRA) resulting in durable remission by stimulating differentiation of the neoplastic clones.^{2,40,18,19}

AML-M3 is a rare condition in animals that to date has only been reported in the literature in a single boar and as a transgenic mouse model.^{7,19} Peer reviewed case reports do not yet exist in dogs, cats, horses, cattle or

sheep. Anecdotal reports suggest the hypogranular variant is more common in domestic animals, possibly presenting with signs referable to a coagulopathy. The severity of clinical disease due to pancytopenia and coagulopathy may hinder a full diagnostic evaluation and contribute to the apparent rarity of this condition.^{37,38}

Acute Myelomonocytic Leukemia (AML-M4)

In acute myelomonocytic leukemia (AML-M4), leukemic cells appear to be of mixed lineage (Fig. 67.1). Three criteria must be met in the bone marrow to achieve a diagnosis of AML-4: $\geq 20\%$ of ANCs must be blast cells, including myeloblasts, monoblasts and promonocytes; $\geq 20\%$ of ANCs must be of monocytic lineage (CD14+, nonspecific esterase [NSE]-positive that is sensitive to fluoride inhibition); and $\geq 20\%$ of ANCs must be of granulocytic lineage (SBB/MPO positive).^{13,15,17,37,38}

Acute Monocytic Leukemia (AML-M5)

Acute leukemias of the monocytic lineage may be divided to the more immature acute monoblastic leukemia (AML-M5a) and the more mature acute monocytic leukemia (AML-M5b). Both subtypes are characterized by $\geq 80\%$ of ANCs being of monocytic origin, and $\geq 20\%$ of ANCs being myeloblasts, monoblasts, or promonocytes (Fig. 67.2). However, in AML-M5a $\geq 50\%$ of all monocytic cells are monoblasts, whereas in AML-M5b $\geq 50\%$ of all monocytic cells are promonocytes.^{2,17,37,38,40} The ALSG did not recommend separating these AML-M5 subtypes because there was poor diagnostic agreement among the group pathologists due to the complexity of cellular identification.¹⁷ Confirmation of monocytic lineage is achieved by demonstrating expression of CD14, and/or fluoride-sensitive NSE. Reportedly,

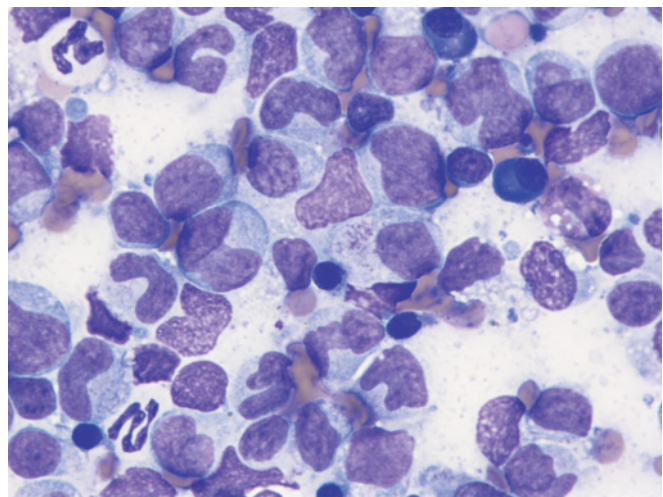


FIGURE 67.1 Acute myelomonocytic leukemia (AML-M4) in bone marrow from a dog. Notice infiltration of $> 20\%$ blast cells with apparent granulocytic and monocytic differentiation but with maturation arrest evident by the paucity of mature forms. Note primary granules in myeloblast near the center of the image. Wright-Giemsa stain; original magnification $\times 1,000$.

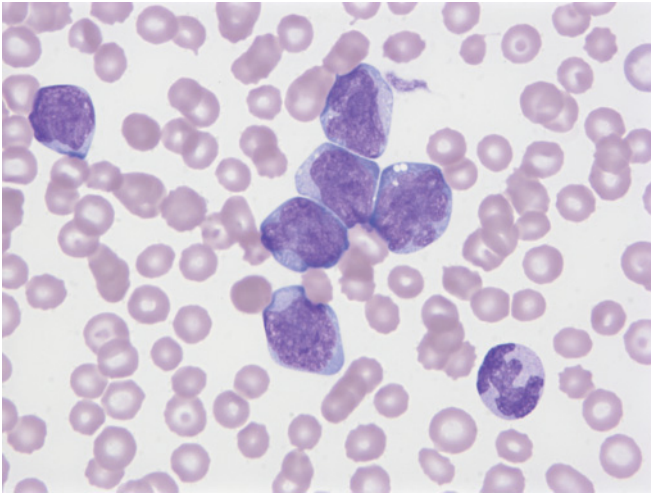


FIGURE 67.2 Acute monoblastic leukemia (AML-M5) in blood smear from a dog. Notice a population of monomorphic blast cells with large convoluted nuclei, lacy to finely stippled chromatin, and moderate to abundant vacuolated, basophilic cytoplasm. The cells resemble monocytic progenitors with no visible cytoplasmic granules. Wright-Giemsa stain; original magnification $\times 1,000$.

AML-M5a is more common in young animals, whereas AML-M5b is more common in mature and older animals. Leukocyte counts vary from $<25,000/\mu\text{L}$ in the early stages of disease, to $>50,000/\mu\text{L}$ terminally.^{13,17,37,38}

Acute Erythroleukemia (AML-M6)

The acute erythroleukemias are characterized by the presence of greater than 50% erythroid precursors in the bone marrow and can be further divided into two groups based on the blast composition (Fig. 67.3). In acute erythroleukemia (AML-M6a) there is myeloblastic predominance and $\geq 20\%$ of all NECs in the bone marrow are myeloblasts. AML-M6a is the type of erythroleukemia most commonly observed in human patients. In contrast, acute erythroleukemia with erythroid predominance (AML-M6b) is characterized by a prominent rubriblastic component. In AML-M6b, $\geq 20\%$ of ANC in the marrow are myeloblasts and rubriblasts. AML-M6b is the type of erythroleukemia most commonly observed in veterinary patients, with many cases occurring in FeLV-positive cats during the FeLV epidemic (see Chapter 62). In both subtypes of AML-M6, left shifting and dysplasia of the erythroid lineage may be evident. The total nucleated cell count in the blood is commonly elevated, sometimes markedly, and may contain blast cells of the erythroid and myeloid lineage as well as other dysplastic erythroid precursor cells.^{2,12,13,16,17,28,37,38,40}

Acute Megakaryoblastic Leukemia (AML-M7)

Like other types of AML, acute megakaryoblastic leukemia (AML-M7) is diagnosed on the basis of $\geq 20\%$ blast

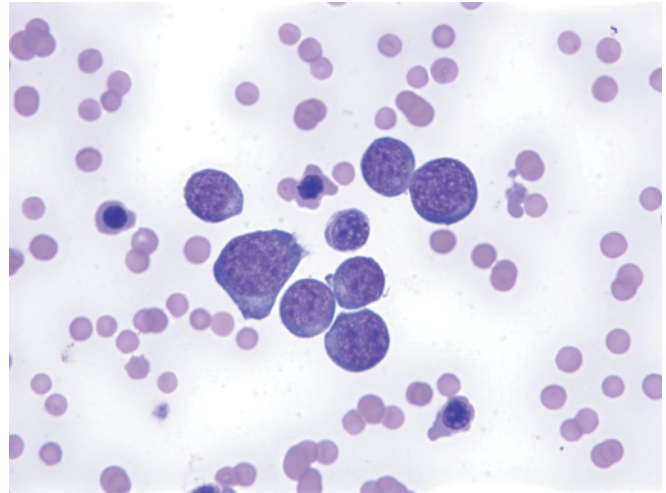


FIGURE 67.3 Erythroleukemia (AML-M6b) in blood smear from a cat. Notice a slightly pleomorphic population of cells that resemble erythroid progenitors, including erythroblasts, prorubricytes, rubricytes, and metarubricytes. There is apparent asynchronous maturation of these cells, and there are fewer than expected mature RBCs, WBC, and platelets. Wright-Giemsa stain; original magnification $\times 1,000$.

cells in bone marrow. At least 50% of the blast cells must be megakaryoblasts. While features such as cytoplasmic blebbing, increased amounts of cytoplasm, and binucleation or multinucleation may be observed in megakaryoblasts, immunologic markers and cytochemical stains are essential for lineage determination. Though nonspecific, megakaryoblasts will often stain positive for acetylcholinesterase (AChE), periodic acid Schiff (PAS), and alpha-naphthylacetate esterase (ANAE) on cytochemistry.^{2,13,20,37,38,40} Expression of CD41, which represent the glycoprotein (Gp) IIb and GpIIIa subunits (i.e. platelet integrin receptor for fibrinogen and factor VIII-related antigen) are considered the most specific lineage identifiers.³⁶ Additional features that may be observed in AML-M7 include thrombocytopenia and sclerotic bone marrow, which may make obtaining a bone marrow aspirate difficult.^{37,38}

Species specific Information

All AML subtypes have been reported in veterinary species. As mentioned previously, published reports of AML-M3 only exist in a boar and a transgenic mouse model, but its occurrence cannot be excluded in other veterinary species.^{7,19} The most commonly reported myeloid leukemias in the dog and cat appear to be the acute myeloid, myelomonocytic, and monocytic leukemias.^{13,17,37,38} Erythroleukemia has been reported in both the dog and cat; however, reports and incidence in cats were more frequent before the FeLV epidemic was controlled. Acute myelomonocytic leukemia is believed to be the most common myeloid leukemia in horses.^{5,9,25,31} Sporadic reports of AML in other mammalian species exist, including a recent report of AML with multilineage dysplasia in an alpaca.³⁵ Finally, retroviral induced

AML has been reported in poultry, primarily in conjunction with the avian leukosis/sarcoma virus (see Chapter 62). Myeloid leukosis in poultry has recently been attributed to the newly identified J envelope subgroup of the slowly transforming ALSVs.²⁹

FUTURE DIRECTIONS IN VETERINARY MEDICINE

Complete immunophenotypic, cytochemical, and (rarely) ultrastructural characterization of all leukemic processes are necessary to determine the true incidence of AML in veterinary species. Other modalities that may allow for more precise and detailed characterization include cytogenetics, gene expression and micro-RNA profiling, proteomics, and stem cell biology (see Chapters 3 and 148). Additionally, recent studies have identified evolutionarily conserved chromosomal aberrations that link several canine hematologic malignancies in dogs to their human counterparts. These findings imply that the neoplasms are pathologically comparable and suggest that cellular and molecular analysis of canine leukemias may allow for improved characterization, classification, prognostication, and treatment.^{6,23}

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Mast Cell Cancer

CHERYL LONDON

Mast Cell Tumor in the Dog

- Incidence, Signalment, Etiology
- History and Clinical Signs
- Diagnosis
- Staging
 - CBC, biochemistry profile, urinalysis
 - Buffy coat smear
 - Bone marrow aspiration
 - Lymph node aspiration
 - Evaluation of the abdominal and thoracic cavities
 - Clinical staging system for canine MCTs
- Prognostic Factors
 - Histologic grade
 - Clinical stage
 - Anatomic location
 - Growth rate
 - Breed
 - Markers of proliferation
 - Kit mutations

Treatment

- Surgery
- Radiation therapy
- Chemotherapy
 - Corticosteroids
 - CCNU (lomustine)
 - Vinca alkaloids
 - Kit inhibitors
- Supportive Care
 - H2 antagonists
 - H1 antagonists
- Feline Mast Cell Tumors
 - Cutaneous Feline MCT
 - Splenic (Visceral) MCT
 - Feline Intestinal MCT
- Equine Mast Cell Tumors

Acronyms and Abbreviations

AgNORs, argyrophilic nucleolar staining organizing regions; CBC, complete blood count; GI, gastrointestinal; ITD, internal tandem duplication; MCT, mast cell tumor; MI, mitotic index; PCNA, proliferating cell nuclear antigen.

MAST CELL TUMOR IN THE DOG

Incidence, Signalment, Etiology

The mast cell tumor (MCT) is the most frequent skin tumor of the dog, and the second most frequent malignant tumor noted in the canine population. While MCTs are usually found in older dogs (mean age 8–9 years), they have also been reported in younger dogs and there is no apparent sex predilection. Several breeds appear to be at risk for the development of MCT including dogs of bulldog descent (boxer, Boston terrier, English bulldog, pug), Labrador and golden retrievers, cocker spaniels, Schnauzers, Staffordshire terriers, Beagles, Rhodesian ridgebacks, Weimeraners, and Sharpeis.³⁴ The etio-pathogenesis of MCTs in the dog is unknown. The

increased incidence of MCTs in certain breeds suggests the possibility of an underlying genetic cause and studies are ongoing to identify putative genetic risk factors. Interestingly, while dogs of bulldog ancestry are at higher risk for MCT development, it is generally accepted that MCTs in these dogs are more likely to be benign. Pugs develop multiple mast cell tumors that behave in a benign fashion.²⁹ In contrast, anecdotal evidence suggests that Sharpeis develop MCTs that are biologically aggressive.

Several authors have recently identified the presence of Kit mutations in dog MCTs and these result in uncontrolled signaling.^{10,22,25,50} In the majority of affected dogs, the Kit mutations consist of internal tandem duplications (ITDs) in the juxtamembrane domain of Kit (encoded by exons 11–12). This region of Kit is

responsible for negatively regulating receptor activation and evidence suggests that the ITDs disrupt the structure of this domain, resulting in a loss of this function. Up to 30% of all dog MCTs may carry Kit mutations and these have been shown to be significantly associated with tumor grade: mutations are rarely identified in well-differentiated tumors while approximately 35% of poorly differentiated tumors carry an ITD.^{10,51} The Kit mutations are not germ-line in nature (i.e. are not inherited) and, therefore are not responsible for the observed breed predispositions to the development of MCTs. However, they do represent a target for therapy.

History and Clinical Signs

Most MCTs in the dog occur in the dermis and subcutaneous tissue.² However, primary MCTs may present in other sites such as the oral cavity, nasopharynx, larynx, and gastrointestinal (GI) tract.³⁵ Visceral MCT, involving the spleen, liver, and/or bone marrow (often referred to as disseminated mastocytosis), is usually the result of systemic spread of an aggressive primary cutaneous MCT, although it can occur as an independent syndrome.³⁷ Cutaneous MCTs usually occur as solitary nodules, although roughly 10% to 15% of dogs will present with multiple tumors.¹⁶ Approximately 50% of cutaneous MCTs occur on the trunk and perineal region, 40% on limbs, and 10% on head and neck.¹⁶ Perhaps most importantly, the clinical appearance of MCTs can vary widely and can resemble many other neoplastic and non-neoplastic lesions. Mast cell tumors that arise in subcutaneous tissue are frequently poorly circumscribed and may resemble lipomas, sometimes delaying diagnosis. In general, MCTs that are slow growing and present for at least 6 months are more likely to behave in a benign manner, while those that are rapidly growing large tumors are more likely to behave in a malignant manner.²

Clinical signs of MCTs are largely due to release of histamine, heparin and other vasoactive amines. Mechanical manipulation of the tumor during physical examination can induce degranulation leading to erythema and wheal formation (termed Darrier's sign) and occasionally, an owner will report that the tumor appears to change in size over short periods of time. Gastrointestinal ulceration is also a potential complication of MCTs; between 35% and 83% of dogs with MCTs that underwent necropsy had evidence of gastric ulcers, and plasma histamine concentrations were found to be elevated in dogs with MCT.¹⁷ Elevated histamine levels presumably lead to stimulation of H2 receptors on parietal cells, excessive gastric acid production, and development of ulcers.

Diagnosis

Cytologic evaluation of fine needle aspirates is probably the easiest method to diagnose MCT. Poorly differentiated malignant mast cells may contain few, if any, granules in which case special stains (toluidine blue, Giemsa) may be necessary to observe granules. However, exci-

sional biopsy is required for actual histologic grading of the tumor. If cytologic diagnosis proves difficult, a needle or punch biopsy of the tumor can be obtained before surgery. This is preferable to a larger incisional biopsy, because local release of mast cell mediators may inhibit healing, resulting in excessive bleeding.

Staging

CBC, Biochemistry Profile, Urinalysis

Dogs with MCTs may be anemic due to gastrointestinal bleeding.

Buffy Coat Smear

It was originally believed that while the buffy coat smear was not a sensitive test, it was specific for mast cell neoplasia. However, it is now clear that this is not the case, because dogs with many different kinds of disease, including pneumonia, parvovirus, pancreatitis, skin disease and GI diseases may have mast cells circulating in the blood. Therefore, this test is no longer routinely performed in the staging of MCT patients.

Bone Marrow Aspiration

In the normal bone marrow, mast cells are found infrequently. In a recent report evaluating 157 dogs with MCTs, the incidence of bone marrow infiltration at initial staging was only 2.8%.¹¹ While the presence of bone marrow involvement is indicative of systemic mast cell disease, it is usually easier to find evidence of systemic involvement in other organs (liver, spleen). Therefore, routine bone marrow aspiration is not recommended for most MCT patients.

Lymph Node Aspiration

All regional lymph nodes should be examined for signs of enlargement and suspicious nodes should be aspirated for cytologic examination. Because metastatic nodes may palpate within normal size, it is recommended that all accessible regional lymph nodes be aspirated. If necessary, ultrasound can be used to localize lymph nodes for aspiration. Malignant mast cells in metastatic lymph nodes are frequently found in clusters/aggregates rather than singly, aiding in a diagnosis of metastasis. If possible, lymph node aspiration should be performed before surgery, because post-operative inflammation can result in mast cell migration to local nodes and thus confuse the interpretation.

Evaluation of the Abdominal and Thoracic Cavities

Thoracic radiographs may be included as part of staging, although pulmonary involvement is uncommon. Abnormalities reported include lymphadenopathy (sternal, hilar), pleural effusion, and anterior mediastinal masses, although these are rare. Evaluation of the

TABLE 68.1 Proposed Clinical Staging for Canine Dermal Mast Cell Tumors

Stage	Tumor(s)	Regional LN	Metastasis
IA	1 tumor, confined to skin, <3cm, well circumscribed	Negative	Negative
IB	>1 tumor, confined to skin, <3cm, well circumscribed, interlesional distance >10cm	Negative	Negative
II	1 or more skin tumors, either >3cm or ill circumscribed or ulcerated or with satellites	Negative	Negative
III	Any	Positive	Negative
IV	Any	Any	Positive

A, without clinical signs; B, with clinical signs.

abdominal cavity is important in dogs with MCTs, as spread to the liver and spleen and abdominal lymph nodes may be noted. It is recommended that fine needle aspiration of the liver and spleen be performed if abnormalities are detected during ultrasound examination, or if the dog possesses negative prognostic indicators (i.e. rapidly growing tumor or evidence of lymph node metastasis.¹⁵ For typical uncomplicated solitary MCTs, cytologic evaluation of an ultrasonographically normal liver or spleen was not found to be a clinically useful staging tool.

Clinical Staging System for Canine MCTs

Based on recent clinical studies, a revised staging system for dermal MCTs has been proposed (Table 68.1).

Prognostic Factors

Histologic Grade

The histologic grade of a MCT is determined after excisional biopsy of the tumor. It is the most consistent and reliable prognostic factor and correlates significantly with survival, but it will not predict the behavior of every MCT. Furthermore, there is disagreement about tumor grading among pathologists; in one study there was significant variation among pathologists in grading the MCTs ($P < 0.001$), although this was found to be less so if all pathologists strictly employed the system described by Patnaik.^{34,35}

Grade 1: These MCTs are considered to behave in benign manner and complete surgical excision is usually curative.^{2,34}

Grade 2: These represent at least 45% of all MCTs reported and their biologic behavior is more difficult to predict.^{2,16,34} Many dogs are cured with complete excision of a Grade 2 MCT, and radiation therapy following incomplete excision of solitary Grade 2 MCTs can cure more than 80% of affected patients.³⁶ However, it is important to note that Grade 2 MCTs have the ability to spread to local lymph nodes, as well as distant sites, and a proportion of dogs that

undergo definitive therapy (surgery and radiation) may go on to develop metastatic disease. Furthermore, some dogs that present with Grade 2 MCTs will already have evidence of metastatic disease making appropriate staging important. Given the wide variation in biologic behavior among Grade 2 tumors, there is now an effort to identify subcategories of Grade 2 tumors using additional prognostic indicators described below.

Grade 3: These represent between 20% and 40% of all MCT reported.^{2,16,34} They often behave in a biologically aggressive manner, exhibiting metastasis early on in the course of disease. The mean survival time of dogs with Grade 3 MCT has been reported as 18 weeks when treated with surgery alone.² In one study, the percentage of dogs with Grade 3 MCTs surviving at 1500 days was reported as 6%, and in another study, the percentage of dogs surviving at 24 months was 7%, indicating that these tumors are particularly malignant.³⁴ With the recent addition of post-operative chemotherapy, survival times of Grade 3 MCT patients may be improved. Lastly, evidence suggests that radiation therapy of incompletely excised Grade 3 MCTs can result in prolonged tumor control (median disease free interval of 27.7 months).¹⁴

Clinical Stage

Recent evidence suggests that the historical staging system for MCTs is not reflective of tumor biology, and a new system has been proposed (Table 68.1). In two studies, the presence of mast cells in the regional lymph node was a negative prognostic factor for survival and disease-free interval.²⁰ However, an additional study revealed that dogs with Grade 2 tumors and lymph node metastasis treated with radiation post-surgery achieved long-term survival.⁸ Other studies have shown that dogs with Grade 2 MCTs with lymph node metastasis may have a good prognosis if the affected lymph node is removed and adjuvant chemotherapy is administered.^{6,46} Lastly, while it would seem intuitive that dogs with multiple cutaneous MCTs would experience a worse outcome compared to dogs presenting with a solitary MCT, two separate studies have demonstrated that the presence of multiple tumors does not affect prognosis.³²

Anatomic Location

MCTs that develop in the oral cavity, nail bed, inguinal, preputial, and perineal regions were originally reported to behave in a more malignant fashion regardless of histologic grade.¹³ Two reports now demonstrate that at least for MCTs in the inguinal, preputial, and perineal regions this is probably not true.⁵ However, MCTs located in the muzzle are biologically aggressive tumors with higher regional metastatic rates than previously reported for MCT in other sites.¹³ MCTs that originate in the viscera (GI tract, liver, spleen) or bone marrow have a grave prognosis.⁴⁵

Growth Rate

Tumors present for long periods of time are more likely to be benign. In one study, 83% of dogs with tumors present for longer than 28 weeks before surgery survived for at least 30 weeks, compared to only 25% of dogs with tumors present for less than 28 weeks.²

Breed

Boxer and pug dogs have a high incidence of MCTs, but these tend to be more well differentiated and carry a better prognosis.^{2,29} However, every MCT should be treated as potentially malignant, regardless of breed.

Markers of Proliferation

Several proliferative indices have been evaluated in an attempt to predict the outcome of canine MCTs. Perhaps the most useful is Ki-67, a protein found in the nucleus, the levels of which appear to correlate with cell proliferation. In one study, the mean number of Ki-67 positive nuclei was significantly higher for dogs that died of MCTs than for those that survived. For dogs with Grade 2 tumors, the number of Ki-67 positive nuclei was significantly associated with outcome. This was recently confirmed by an additional study that demonstrated the Ki-67 score can be used to divide Grade 2 MCTs into two groups with markedly different expected survival times.⁴² A recent study showed that mitotic index (MI, number of mitoses per 10 high-power fields) may be useful for predicting the biologic behavior of canine MCTs.⁴¹ When dogs presenting with metastatic disease were excluded from analysis, those with tumors possessing a MI ≤ 5 had a median survival time of 80 months, compared to 3 months for those possessing a MI > 5 , suggesting that MI is a strong predictor of overall survival for dogs with MCTs. Other proliferation markers such as assessment of argyrophilic nucleolar staining organizing regions (AgNORs) and PCNA have been used to try to determine biologic behavior of MCTs, although these may not be as reliable.³ One study sought to establish a new grading scheme for MCTs using Kit immunohistochemical staining patterns as an indicator of tumor aggressiveness but it did not correlate well with survival time.⁴⁹ Lastly, investigators attempted to correlate histologic grading of mast cell tumors with a combined Ki67/PCNA/AgNOR/Kit immunohistochemical scoring.¹ No significant correlation was found for Kit staining and MCT grade, but high Ki67/PCNA/AgNOR scores all positively correlated with tumor grade (i.e. higher scores for higher grade). This study suggests that proliferation indices increase with increasing grade and are ultimately reflected in the eventual biologic behavior of the tumor.

Kit Mutations

As previously mentioned, mutations in Kit have been found in canine MCTs and research suggests they are

TABLE 68.2 Schematic for Treatment of Canine Mast Cell Tumors

Grade I	
Complete	No further therapy
Incomplete	Wider excision or radiation therapy if surgery not possible; may consider no further therapy
Grade II	
Complete	Chemotherapy only if negative prognostic factors present
Incomplete	Wider excision or radiation therapy if surgery not possible; may consider no further therapy if there are no negative prognostic indicators; chemotherapy if negative prognostic factors present
Grade III	
Complete	Chemotherapy
Incomplete	Chemotherapy +/- radiation therapy

associated with an increased risk of local recurrence, metastasis, and death of affected dogs.^{10,51}

Treatment

See Table 68.2.

Surgery

Historically, it has been recommended that the margins need to be at least 3 cm in each direction; deep margins are as important as the lateral margins. Recent studies demonstrated that all Grade 1 MCTs were completely excised with 1 cm of normal tissue around the MCT (lateral margins) and one fascial plane included in the excision (deep margin).^{5,26,44} With respect to Grade 2 MCTs in both studies, 75% and 68% were completely excised with a 1 cm lateral margin and one fascial plane deep, while 100% and 89% of Grade 2 MCT were completely excised with a 2 cm lateral margin and one fascial plane deep. Neither of the studies evaluated Grade 3 MCTs. Because tumor grade is usually not known prior to surgery, it appears prudent to still recommend a 3 cm lateral margin and one fascial plane for the deep margin when feasible. All of the excised tissue should be submitted and margins should be labeled so the pathologist is able to specifically identify any areas of incomplete excision. However, even histologically clean margins do not guarantee that a tumor will not recur. In one study, 83% of dogs with Grade 1 MCT, 44% of dogs with Grade 2 MCT, and 6% of dogs with Grade 3 MCT were alive 1500 days after surgical excision.³⁴ A proportion of Grade 2 tumors that are incompletely excised will not recur post surgery. In a recent report, the estimated proportions of Grade 2 tumors that recurred locally at 1, 2, and 5 years were 17.3%, 22.1%, and 33.3% respectively.⁴³ Eleven (39.3%) dogs developed MCT at other cutaneous locations and median overall survival was 1426 days.

Radiation Therapy

Substantial data suggest that radiation therapy is effective in eliminating remaining microscopic disease after incomplete excision of Grades 1 and 2 MCT (greater than 90% 3 year control rate).^{20,36} Radiation therapy also was found to be useful for dogs with MCTs metastatic to local lymph node. In this report, administration of radiation to the incompletely excised tumor and to affected lymph node resulted in a median disease-free survival of 1240 days.⁸ With respect to incompletely excised Grade 3 tumors, evidence suggests that radiation therapy can result in prolonged tumor control (median disease free interval of 27.7 months) although dogs with tumors greater than 3 cm before surgery did not fare as well.¹⁴ It is likely that multi-modality therapy (i.e. combining surgery, radiation, and chemotherapy) would enhance this observed survival time. Radiation therapy has also been used to treat solid MCTs when surgery is not an option. Varying degrees of success have been found; in one study, a 60% 1-year control rate was obtained in dogs with non-resectable Grades 1–3 MCTs on the head or limb treated with prednisone and radiation. Coarse fractionated radiation has been used to treat dogs with non-resectable bulky, high grade MCTs, although it is important to note that systemic effects of degranulation following radiation may lead to vomiting, hypotension, and death.

Chemotherapy

The use of adjuvant chemotherapy is indicated after excision of Grade 3 MCTs, metastatic MCTs, non-resectable high-grade tumors, or for any other MCT with negative prognostic indices. While radiation therapy is the treatment of choice for incompletely excised Grades 1 and 2 MCTs, data indicate that post-operative chemotherapy may prevent local recurrence and, therefore should be considered for patients who are not candidates for radiation, if such therapy is not available or if the owners cannot afford the cost of therapy.

Corticosteroids The reported response rate of canine MCT to prednisone is 20–70%, although the study demonstrating the highest response rate was of short duration (median 10 days) precluding assessment of response durability.²⁷ Partial remissions are more common than complete remissions, and at least some of the observed response may be due to a decrease in tumor-associated edema secondary to stabilization of mast cell granules and a reduction in mast cell mediator production.

CCNU (Lomustine) In one study, 8 out of 19 dogs (42%) with measurable MCTs had an objective response to single agent CCNU for a median duration of 77 days.³⁸ Preliminary unpublished data suggest that CCNU given post-surgery, either alone or with prednisone and vinblastine, can significantly prolong survival times of dogs with high grade tumors or tumors with negative prognostic indicators. CCNU can induce hematopoietic and hepatic toxicity including neutrope-

nia, thrombocytopenia, and liver failure. Patients receiving this drug should be monitored closely. In general, CCNU is dosed at 50–70 mg/m² orally every 3–4 weeks. A complete blood count (CBC) and liver panel should be performed before each dose.

Vinca Alkaloids Single agent response rates of vincristine, vinblastine and vinorelbine are 7%, 12% and 13%, respectively, suggesting that vinca alkaloids are not effective as sole agents for the treatment of MCTs.²⁷ Vinblastine has been combined with prednisone in other studies, inducing objective responses ranging from 27% to 47%.⁴⁶ A combination of vinblastine, cyclophosphamide and prednisone resulted in a 64% (7 out of 11) response rate in one study.⁶ The dose of vinblastine is 2–3 mg/m² given intravenously every 1–3 weeks. The major toxicity of this drug is neutropenia (in approximately 5–6% of patients) and occasional gastrointestinal upsets. It is frequently used in an alternating manner with CCNU.

Kit Inhibitors Orally bioavailable small molecule inhibitors of Kit (SU11654 [Palladia] and AB1010 [Kinavet]) have recently been demonstrated to have activity against canine MCT.^{22,23,24} In a placebo-controlled randomized study, response rate in Palladia-treated dogs was 37.2% versus 7.9% in placebo-treated dogs.²⁴ Of 58 dogs that received Palladia after placebo escape, 41.4% experienced an objective response. The overall response rate for dogs in this study receiving Palladia was 42.8%. Additionally, the commercially available Kit inhibitor imatinib mesylate (Gleevec) has been used to treat canine MCTs. A recent study demonstrated some response to therapy in 10 of 21 dogs treated with imatinib; the objective response rate was 100% in dogs whose MCTs possessed a Kit ITD.¹⁷ Another study reported partial responses to therapy in three dogs with systemic mast cell disease treated with imatinib.²⁶ However, it is important to note that imatinib can cause severe idiosyncratic hepatotoxicity and is extremely expensive, thereby limiting its use.

Supportive Care

H2 Antagonists

Mast cell tumors may cause GI ulceration because histamine stimulates gastric acid production by parietal cells. This is especially relevant for dogs with evidence of systemic involvement. To prevent this, any of the standard H2 antagonists (cimetidine, ranitidine, or famotidine) should be administered to affected dogs. Alternatively, proton pump inhibitors such as omeprazole may be utilized; these inhibitors are probably more useful in the setting of gross mast cell disease where standard H2 antagonists may be less effective.

H1 Antagonists

Massive mast cell degranulation can lead to hypotensive shock and death. Therefore, all patients with gross

mast cell disease, or evidence of systemic involvement should be placed on the H1 antagonist diphenhydramine.

FELINE MAST CELL TUMORS

Mast cell neoplasia in the cat occurs in three basic forms: cutaneous MCT, splenic mast cell disease (sometimes referred to as lymphoreticular) and intestinal MCT. The biologic behavior of these diseases is strikingly different, so they will each be considered separately.

Cutaneous Feline MCT

Cutaneous MCTs represent the second most commonly encountered cutaneous tumor in the cat.³⁰ Two forms have been reported: mastocytic MCTs that appear histologically similar to those found in dogs, and histiocytic MCTs, a rare variant known to spontaneously regress, that possess features of histiocytic cells.⁹ In general, feline cutaneous MCTs are solitary, raised, firm, hairless, well-circumscribed dermal nodules between 0.5 and 3 cm in diameter.^{7,30} Occasionally, the surface of the tumor may be ulcerated; some tumors will present as plaque-like lesions, and tumors can be multiple.⁷ These tumors are most likely to occur on the head and neck (frequently involving the pinna near the base of the ear), trunk, and limbs.^{7,30} Affected cats usually do not exhibit clinical signs of disease other than pruritus.

As with canine tumors, feline MCTs are usually easily diagnosed by cytologic examination of fine needle aspirates. Cats with cutaneous MCT should be evaluated for evidence of additional tumors, as well as potential splenic involvement as a recent study found that some cats with multiple cutaneous MCTs also have splenic disease.²¹ In addition, a minimum database is recommended, along with careful examination of local lymph nodes for evidence of lymphadenopathy.

With respect to the typical mastocytic variant of feline MCT, they are usually categorized as either compact (representing 50–90% of all cases) or diffuse (histologically anaplastic).⁷ Several studies have demonstrated that well-differentiated compact tumors tend to behave in a benign manner and metastasis is uncommon.^{21,31} In contrast, anaplastic tumors may have a high MI, and marked cellular and nuclear pleomorphism, with infiltration into subcutaneous tissues. These behave in a more malignant manner, metastasizing to local lymph nodes, spleen, blood, and bone marrow. However, a more recent study evaluated pleomorphic cutaneous MCTs from 15 cats and found that the majority were behaviorally benign; only one cat was euthanized due to disease progression.¹⁹ Interestingly, in both studies, tumors with a high MI were most likely to recur or exhibit metastasis, suggesting that this feature is useful for predicting biologic behavior.

The definitive treatment for cutaneous feline MCT is surgical excision. In a series of 32 cats with cutaneous MCT, five recurred after surgical excision, but no cats

died of the disease. In this study, completeness of excision and histopathologic factors, such as nuclear pleomorphism and MI, were not associated with tumor recurrence.³¹ Other reports have demonstrated local recurrence rates after excision of between 0% and 24%.^{7,19,21} Systemic spread of cutaneous tumors has been reported in 0% to 22% of cases, although those that metastasized may have been anaplastic tumors.^{19,31} As previously discussed, those tumors with a high MI are more likely to recur post-surgery or metastasize.¹⁹

Radiation therapy may be considered for tumors that are incompletely excised. A recent study demonstrated that feline cutaneous MCTs treated with strontium 90 irradiation exhibited a 98% control rate, with median survival times greater than 3 years.⁴⁸ Limited information exists concerning the utility of chemotherapy in cats with MCT. It is generally believed that feline MCTs are less responsive to prednisone than their canine counterparts. Responses to CCNU have been reported in cats: of 20 cats with cutaneous MCTs, 10 exhibited complete ($n = 2$) or partial ($n = 8$) response to CCNU.³⁹ Some investigators have utilized a combination of prednisone and chlorambucil to treat metastatic or multiple tumors. This is generally well-tolerated, although its effectiveness is unclear.

Splenic (Visceral) MCT

This form of neoplastic mast cell disease is also referred to as visceral or lymphoreticular MCT. It is the most frequent disease of the spleen of cats, affecting older animals (mean age 10 years) with no sex or breed predilection.^{7,21} While the spleen is the primary site affected by this disease, other organs may also be involved including the liver, mesenteric lymph nodes, bone marrow, lung, and intestine.^{7,12} Circulating mast cells may be found in many cases and pleural and/or peritoneal effusions may be noted.^{7,12} The majority of cats with splenic MCTs do not have a history of cutaneous MCT, although recent evidence suggests that cats with multiple cutaneous MCTs may also have splenic involvement.²¹ Cats with splenic MCT frequently present with signs of systemic illness including vomiting, anorexia, and weight loss.^{7,12} Dyspnea may be evident if pleural effusion is present. Abdominal palpation usually reveals a markedly enlarged spleen and/or liver. Clinical signs associated with release of mast cell mediators include GI ulceration, hemorrhage, hypotensive shock, and labored breathing.

Cats with suspected splenic MCT should undergo a standard clinical evaluation including a CBC, chemistry profile, urinalysis, abdominal ultrasound, and thoracic radiographs. Unlike the cutaneous form of MCT in cats and dogs, splenic MCT in the cat is frequently associated with circulating mast cells and up to 50% of cats will have evidence of bone marrow infiltration by mast cells.^{7,12} It is important to note that the finding of multi-organ involvement does not necessarily indicate a poorer prognosis. Anemia is also a frequent hematologic finding, with eosinophilia less likely to be observed.^{7,12} Lastly, in one report of 43 cats with splenic

mast cell disease, 90% had an abnormal coagulation profile, although there was no evidence of clinical bleeding.¹²

Splenectomy is the treatment of choice for cats with splenic MCT, even if other organ involvement is noted. Pre-treatment with H1 and H2 blockers before therapy is indicated; intraoperative death may occur due to the release of mast cell mediators. Median survival times of 12–19 months after splenectomy alone have been reported in cats with splenic MCT with bone marrow/blood involvement.¹² While mastocytosis in the circulation usually does not completely resolve, it does decline significantly and cats experience good quality of life for long periods of time. Cats should be followed post-operatively with buffy coat smears, as a rise in the number of mast cells in the blood may indicate disease progression. Anorexia, significant loss of body mass and the male sex were found to be negative prognostic indicators in one study.¹² Adjunctive chemotherapy with prednisone, lomustine, and/or chlorambucil has been attempted in a limited number of cases, but clinical value is uncertain. Recently, a cat with splenic mast cell disease and systemic mastocytosis was treated with the Kit inhibitor imatinib and exhibited a significant response to therapy including complete resolution of tumor masses and marked reduction in circulating mast cells.¹⁸

Feline Intestinal MCT

Mast cell tumors represent the third most common intestinal tumor in the cat, with lymphoma and adenocarcinoma first and second, respectively.⁷ Most cats have a history of vomiting, diarrhea and anorexia, and a solitary palpable abdominal mass is usually evident on physical examination.⁷ As metastasis is common with this disease, enlarged mesenteric lymph nodes and/or hepatomegaly may also be noted. A peritoneal effusion may be present, and this frequently contains mast cells and eosinophils. Diagnosis may be made by fine needle aspiration of the mass or involved organs. As with splenic MCT, these cats should be staged by performing a CBC, chemistry profile, urinalysis, thoracic radiographs, and abdominal ultrasound. Buffy coat smear and bone marrow aspiration may also be performed, although unlike splenic MCT, intestinal MCT rarely involves these organs. However, it may be useful to distinguish splenic MCT with intestinal involvement from the distinct syndrome of intestinal MCT.

Surgery is the treatment of choice for intestinal MCT. Wide surgical margins are necessary (5–10 cm) as the tumor frequently extends well beyond the observable gross disease.⁷ The historical survival times of cats with intestinal MCT are poor, as metastasis is common at the time of diagnosis. Limited information regarding the use of chemotherapy in these cases is available, although anecdotal responses to lomustine and chlorambucil have been reported. Recently, two cats had objective responses (one complete, one partial) after treatment with lomustine.³⁹

EQUINE MAST CELL TUMORS

Mast cell tumors are known to occur in the horse and the majority are cutaneous in nature.^{28,40} The largest series of tumors reported evaluated 32 cutaneous MCTs and found that 22 of 25 did not recur for up to 6 years after removal, two recurred at the surgical site, and one spontaneously regressed within 3 months after biopsy.²⁸ The majority of published data indicate that equine cutaneous MCTs are benign in behavior, seldom recurring after surgical excision.

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B Cell Tumors

V.E. TED VALLI

Precursor B Cell Neoplasms

B Cell Acute Lymphoblastic Leukemias (ALL) L1

Clinical features

Acute Lymphoblastic Leukemia L2

Acute Lymphoblastic Leukemia L3

Mature (Peripheral) B Cell Neoplasms

B Cell Chronic Lymphocytic Leukemias/Small

Lymphocytic Lymphomas

Pathologic features

Clinical features

Plasma Cell Myeloma (see Chapter 70)

B Cell Prolymphocytic Leukemias

Clinical features

Pathologic features

Lymphoplasmacytic Lymphoma

Pathologic features

Marginal Zone Lymphoma

Clinical presentation

Pathologic features

Clinical features

Mantle Cell Lymphoma

Pathologic features

Clinical features

Follicular Lymphoma

Clinical presentation

Pathologic features

Clinical features

Diffuse Large B cell Lymphomas

Pathologic features

Clinical features

Burkitt's Lymphoma

Pathologic features

Clinical features

Acronyms and Abbreviations

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CB, centroblastic; CHOP, cyclophosphamide, vincristine, doxorubicin, prednisone; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B cell lymphoma; EBV, Epstein-Barr virus; FAB, French-American-British; FL, follicular lymphoma; IB, immunoblastic; ICAM, intercellular adhesion molecule; LPL, lymphoplasmacytic lymphoma; MCL, marginal cell lymphoma; MZL, marginal zone lymphoma; PCR, polymerase chain reaction; PLL, prolymphocytic leukemia; RBC, red blood cell; SLL, small lymphoblastic leukemias; SNP, single nucleotide polymorphism; WBC, white blood cell; WHO, World Health Organization.

Lymphoid tumors have been enigmatic neoplasms for much of the time that they have been diagnosed by pathologists. A problem for clinicians and diagnosticians has always been the basis for distinguishing leukemia from lymphoma. Both diseases were found to have cells of identical immunophenotypes. This difficulty was overcome in the revised WHO classification system that pragmatically linked the diagnosis to the topographical area with the greatest volume of tumor. By this system, if most of the tumor was in bone marrow the disease was termed leukemia and if most was in peripheral tissues it was termed lymphoma.

A further advance in our understanding of lymphomas is that there are always neoplastic cells in circula-

tion with the risk of dissemination related to surface adhesion molecules (ICAMs) that function like the address on an envelope and determine where the cells bearing those markers can adhere to the vessel wall and exit the blood vascular system. In this context, the cells of acute leukemias lack most adhesion sites and tend to remain in circulation. The other extreme is MALT-type lymphomas that may remain so tightly adhered to a single location that they can be successfully managed by surgical removal.

A generality of lymphoid proliferation is that malignancy of poorly differentiated lymphocytes is likely to occur in bone marrow of young individuals and present as leukemia, while malignancy of mature lymphocytes

will likely occur in peripheral tissues of mature humans or animals and present as lymphoma (R. Brunning, personal communication). An interesting exception to that thumb rule is seen in the gamma delta T cell lymphomas of humans, dogs, and cats that arise in intestine and involve the spleen.

When bone marrow is invaded by a hematopoietic neoplasm, some degree of marrow failure will be apparent when 50% or greater volume of marrow is involved with neoplastic cells. Significant clinical cytopenias may be apparent with much less than 50% of marrow involved in some peripheral T cell lymphomas where the neoplastic cells induce suppression of normal marrow cell production. Cats may present with weight loss, vomiting, diarrhea, and inflammatory bowel-type disease with tumor cells usually spreading to spleen

and marrow at the time of diagnosis. Similar conditions seem to occur in dogs; however, neutropenia is not a feature of large granular lymphomas in either dogs or cats.

B and T cell leukemias are classified according to the French-American-British (FAB) system (Table 69.1). With this system the cells of acute lymphocytic leukemia are designated as L1, L2, or L3 types based on cell morphology. This system is less commonly applied today in human medicine because of their reliance on genetics and immunocytochemistry. The FAB system still has relevance in veterinary pathology where early diagnostic interpretation is based on the appearance of leukemic cells in Wright-Giemsa-stained preparations. Differentiating acute myeloid from acute lymphoid leukemias in animals remains a major difficulty. The

TABLE 69.1 Revised European-American Classification of Lymphoid Neoplasia (REAL) 1994^a

B-cell neoplasms

Precursor B-lymphoblastic leukemia/lymphoma

Mature (peripheral) B-cell neoplasms

B-cell chronic lymphocytic leukemia/prolymphocytic leukemia/small lymphocytic lymphoma

Lymphoplasmacytoid lymphoma/immunocytoma

Mantle cell lymphoma

Follicle center lymphoma, follicular

Provisional cytologic grades: I, small cell; II, mixed small and large cell; III, large cell

Provisional subtype: diffuse, predominantly small cell type

Marginal zone B-cell lymphoma, extranodal mucosa-associated lymphoid tissue type (\pm monocytoid B-cells)

Provisional subtype: Nodal marginal zone lymphoma (\pm monocytoid B-cells)

Provisional entity: Splenic marginal zone lymphoma (\pm villous lymphocytes)

Hairy cell lymphoma

Plasmacytoma/plasma cell myeloma

Diffuse large B-cell lymphoma

Subtype: Primary mediastinal (thymic) B-cell lymphoma

Burkitt's lymphoma

Provisional entity: High-grade B-cell lymphoma, Burkitt's-like

T-cell and putative natural killer-cell neoplasms

Precursor T-cell neoplasm

Precursor T-lymphoblastic lymphoma/leukemia

Mature (peripheral) T-cell and natural killer-cell neoplasms

T-cell chronic lymphocytic leukemia/prolymphocytic leukemia

Large granular lymphocyte leukemia (LGL)

T-cell type

Natural killer-cell type

Mycosis fungoides/Sézary syndrome

Peripheral T-cell lymphomas, unspecified

Provisional cytologic categories: medium-sized cell, mixed medium- sized and large cell, large cell, lymphoepithelioid cell

Provisional subtypes: hepatosplenic $\gamma\delta$ T-cell lymphoma; subcutaneous panniculitic T-cell lymphoma

Angioimmunoblastic T-cell lymphoma

Angiocentric lymphoma

Intestinal T-cell lymphoma (\pm enteropathy-associated)

Adult T-cell lymphoma/leukemia

Anaplastic large cell lymphoma, CD30+, T- and null cell types

Provisional entity: anaplastic large cell lymphoma, Hodgkin's-like

Hodgkin's lymphoma

Lymphocyte predominance

Lymphocyte-rich (classical Hodgkin's lymphoma)

Nodular sclerosis

Mixed cellularity

Lymphocyte depletion

^aReproduced with permission from Knowles DM, ed. Neoplastic Hematopathology, 2nd ed. Philadelphia: Lippincott Williams and Wilkins, 2001;699, table 19.13.

availability of a myeloperoxidase stain that works in both immunocytochemistry and immunohistochemistry on canine and feline cells has greatly assisted our ability to recognize acute myeloid leukemia (AML).

PRECURSOR B CELL NEOPLASMS

B Cell Acute Lymphoblastic Leukemias (ALL) L1

B cell lymphoblastic leukemias are clonal diseases primarily involving bone marrow that present with mild to moderate levels of leukemic lymphocytosis but can be characterized by leukopenia.^{7,37} The disease is associated with splenomegaly but not with a mediastinal mass seen in T cell-type ALL.

Clinical Features

L1 B cell ALL are relatively infrequent lymphomas usually of young animals.³¹ It is most often recognized in dogs and cats but probably occurs in all mammals. L1-type B cell ALL is a disease of acute onset. Affected animals are in good condition with acute loss of appetite. Organomegaly is usually mild but spleen is symmetrically enlarged and nodal changes are irregular or absent.

Leukemia blast cells are always present in the blood varying between 10,000 and 50,000/ μL and the total white blood cell (WBC) count may be normal or decreased. The key to the diagnosis is the recognition of a homogeneous population of cells with round densely stained nuclei only slightly larger than RBCs. The nucleoli are obscured by the dispersed chromatin and are only recognized if carefully looked for. The cytoplasm is scant and visible over about one-third of the nuclear circumference. Mitoses are usually present in the blood.

Marrow is always involved and there may be some degree of phthysis with reduced erythropoietic cells and megakaryocytes (Fig. 69.1). Hemorrhage is present in marrow and is usually identified on gross examination of marrow. In early stages, lymph nodes may have few changes but, if involved, there will be atrophy of germinal centers with colonization of the inner paracortical areas and medullary cords with cells as described for marrow (Fig. 69.2). With progression and marrow failure, there will be extramedullary hematopoiesis in both medullary cords and sinuses. The spleen will have uniform involvement with resultant rounded borders and may have focal areas of hemorrhagic infarction. There is atrophy of the lymphoid follicles and of periarteriolar lymphoid sheaths with variable sinus colonization (Fig. 69.3). There may be colonization of subendothelial areas of large muscular veins. The liver also may be involved. The involvement is tightly periportal and tends to spread in solid proliferation. Other tissues that may be involved include skeletal muscle, intestine, brain, and testis.

In terms of phenotype, L1 may be of B cell- or T cell-type; not all cells of L1 type will stain with CD3 (T cell

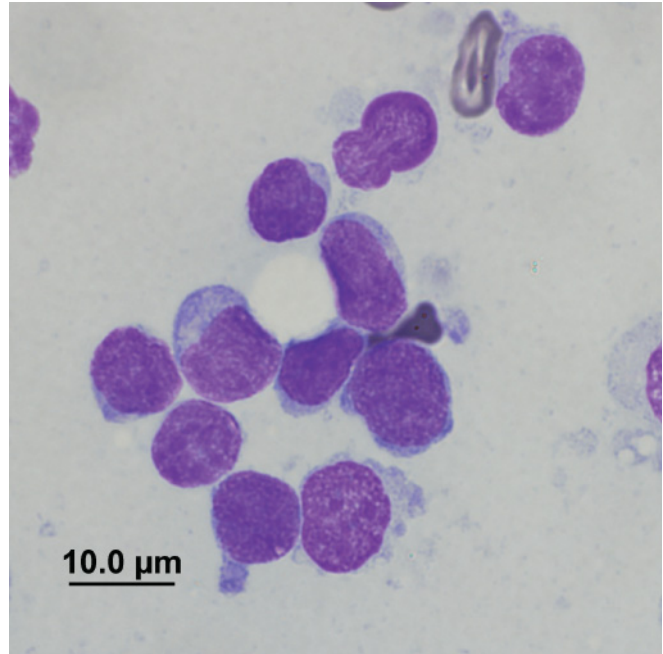


FIGURE 69.1 Bone marrow aspirate from a dog with L1 acute lymphoblastic leukemia. There is phthysis of normal cells and replacement by small neoplastic lymphocytes. Note the size of the nuclei of the smaller cells in comparison to RBCs. The chromatin is densely stained and uniformly distributed with nucleoli not apparent. Cytoplasm is limited to a very narrow ellipse not visible on all cells. The larger cells with more red stained nuclei are the dividing cells. Wright-Giemsa stain.

marker) or CD79a (B cell marker), but almost none of them stain with both. Therefore, even slight staining is taken as a specific indication of lineage. These cells stain equally well in cytologic and histologic preparations with CD20 (B cell marker) that is more reliable in cats than CD79a. CD20 will occasionally stain T cells and if the population is questionably marked by both CD20 and CD3 the cells should be considered to be T cells.

In flow cytometric analysis, L1 B cell ALL is identified by CD45-positive blasts of very small cell size. In addition, in human medicine, the B cell blasts are positive for CD10, CD19, and HLA-DR and negative for the T cell marker CD7. In contrast, the L1-type T cells are identified in flow cytometry as small blast cells that are positive for CD7 and are doubly positive with CD4 and CD8.

Few molecular changes are defined in animals with L1-type ALL. Hyperdiploidy is present in nearly half of human cases occurring in children. The most common chromosomal alteration in children occurs in about 10% of cases and involves translocations and deletions in chromosome 12.

Presumably the benign counterpart cell of origin is a primitive precursor in marrow in the early B cell lineage. Human subsets are identified as pre-B cell ALL and B acute ALL based on the level of immunoglobulin gene expression.

The L1-type ALL is defined as tumors in which the neoplastic nuclei are larger than RBCs but less than

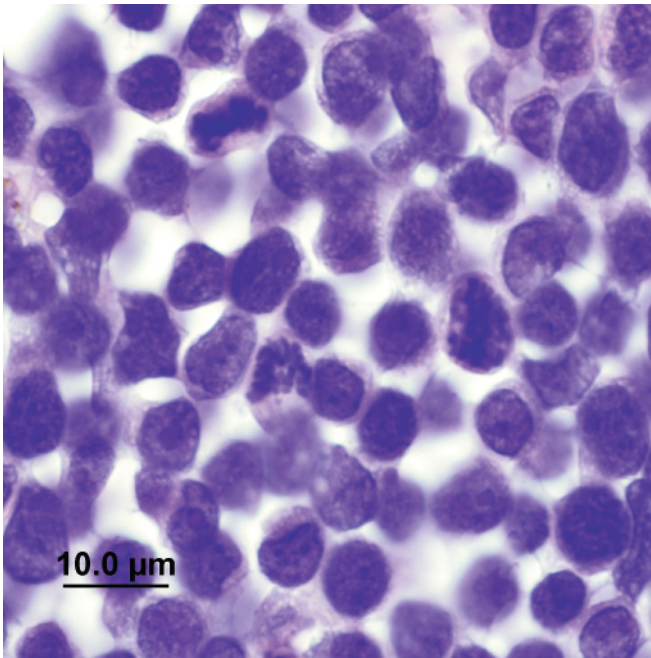


FIGURE 69.2 Lymph node from a dog with L1 acute lymphoblastic leukemia. The nuclei are round and small to intermediate in size with dispersed chromatin. Nucleoli are obscured and only irregularly apparent. There are three mitotic figures in the upper left and two in left and right center. Note that the metaphase chromatin of lymphoblastic lymphoma in histological preparations do not have the sharp outline of chromosomes as is seen in other subtypes of lymphoma. Hematoxylin & Eosin stain.

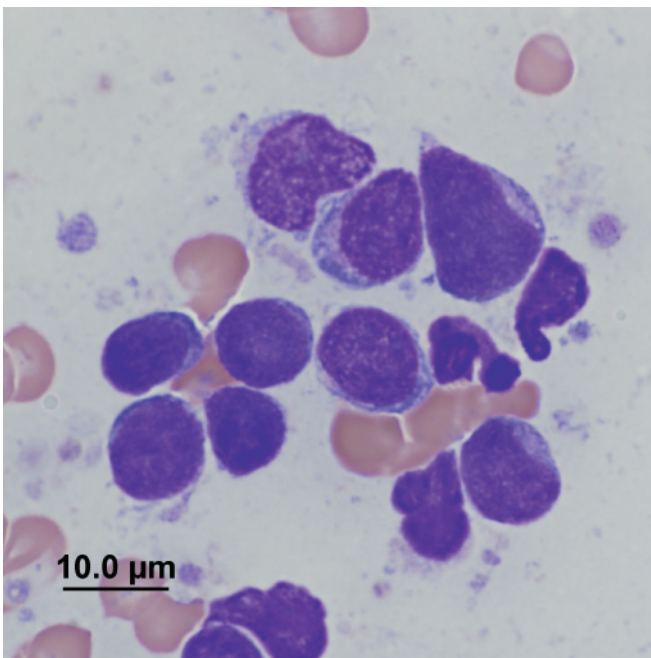


FIGURE 69.3 Splenic aspirate from a dog with L1 acute lymphoblastic leukemia. Note the size of the smaller cells as compared to RBCs with cell type identical to that noted in bone marrow. Wright-Giemsa stain.

1.5 RBC diameters. The chromatin pattern is coarsely stained and may have a coarse cribriform chromatin structure with the nucleoli obscured. This is important because in lymphoblastic lymphoma the chromatin is dispersed and the nucleoli are present but obscured. Additionally, L1-type ALL cells have very little cytoplasm.

In the FAB system the L1 form is essentially the leukemic form of lymphoblastic lymphoma. In histopathologic preparations, both L1 and L2 are virtually indistinguishable from lymphoblastic lymphoma. B cell ALL must be distinguished from T cell ALL that is morphologically identical. B cell ALL is not associated with hypercalcemia or a mediastinal mass both of which occur in a proportion of L1-type T cell ALL. CD3 is the best marker for T cell ALL. The most important topographic finding in T cell ALL is an anterior mediastinal mass.

Staging of B cell ALL in animals is primarily based on the degree of bone marrow involvement and on the presence of cytopenias in the blood. All cases of B cell ALL can be expected to advance rapidly. Treatment usually consists of multi-agent protocols with both B cell and T cell ALL considered to have a very poor prognosis in dogs and cats; aggressive therapy is generally not given to other species. Evaluation of treated cases is essentially monitoring the levels of tumor cells in the blood and monitoring developing cytopenias. Most cases progress to marrow failure.

Acute Lymphoblastic Leukemia L2

B cell lymphoblastic leukemia of L2 type is a clonal disease involving bone marrow that presents with mild to moderate levels of leukemic lymphocytosis but can be characterized by leukopenia. The disease is associated with organomegaly that includes at least moderate involvement of spleen, liver, and lymph nodes, and may involve the kidneys, intestinal tract, and reproductive tract. Most acute lymphoid leukemias of animals are of the L2 ALL type. The L2 type of B cell ALL is seen in the dog, cat, calves, cattle, horses, and laboratory species. In human cases, the L1 and L2 ALL do not appear to be biologically different diseases. Consequently, L2 B cell ALL can be considered to be like L1 B cell ALL.

The L2-type lymphomas are characterized by nuclei that are fully 2 RBCs in diameter and have sharp shallow nuclear indentation. The chromatin pattern is coarsely cribriform with dense staining and occasional chromatin aggregations. These aggregates frequently surround the nucleolus giving it added prominence (Fig. 69.4). Mitoses are usually seen in cytologic preparations. There is a high N:C ratio with cytoplasm seen around no more than half of the nucleus.

In L2 ALL cases without nuclear indentations, the cells resemble AML of the FAB M0 or M1 blast type. Some cases of L2 ALL may be of the large granular lymphocyte type and these tend to have a worse prognosis in humans and it appears that this also applies in cats and dogs. In general the same immunologic char-

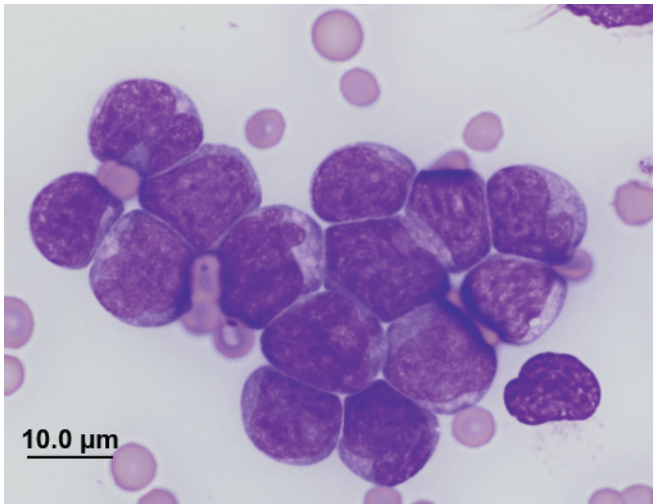


FIGURE 69.4 Blood smear from a dog with L2 acute lymphoblastic leukemia. The nuclei are generally 2–2.5 RBCs in diameter with frequent deep nuclear indentations and irregular rounded projections. The chromatin is deeply stained with irregular formation of dark chromocenters that outline some nucleoli. The cytoplasm is generally minimal in volume without an apparent complete covering of nuclei. There is moderate cytoplasmic basophilia without vacuolation or granulation. Wright-Giemsa stain.

acteristics can be applied to L2 ALL as for L1 ALL with the exception of differences due to cell size and cytoplasmic volume.

Acute Lymphoblastic Leukemia L3

Acute lymphoblastic leukemias of the L3 type are acute clonal neoplasms involving marrow and generally are associated with organomegaly. L3-type ALL occurs in most mammals, being relatively frequent in dog, cat, and cattle, and less common in horses. L3-type ALL cells have fine peripheral cytoplasmic vacuolization that may stain positively for fat (Fig 69.5). The cytoplasm is similar in volume to the L2-type ALL and usually deeply basophilic. Nuclei vary from 2 to 3 RBCs in diameter. The chromatin is deeply stained and may be dense and dispersed or cribriform with some dense chromocenters that may encircle nucleoli that are multiple and moderately well defined. In some individual cases, nuclei mold into oval shapes and there may be deep nuclear indentations particularly in cattle. Mitoses are generally present.

In both cytologic and histologic preparations L3 ALL is not reliably distinguishable from Burkitt-type lymphoma or Burkitt-like lymphoma. The anisokaryosis that is seen in the cytologic preparations of L3-type ALL is also evident in the Burkitt-like lymphomas.

The L3 ALL cells also may be confused with large T cell lymphoma, megakaryocytic leukemia, and monocytic leukemias, all of which may have cytoplasmic vacuolizations. Like L3 ALL, these tumors are negative with myeloperoxidase staining. L3 B cells stain positively for CD79a and CD20 and negatively for CD3. In

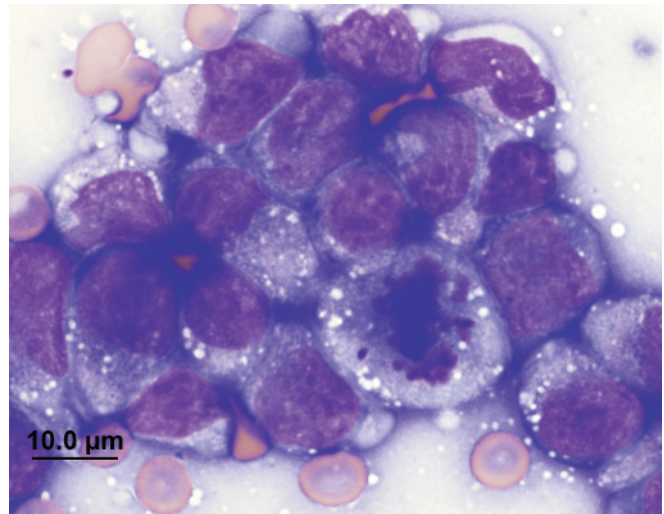


FIGURE 69.5 Lymph node aspirate from a dog with L3 acute lymphoblastic leukemia. The nuclei have mild anisokaryosis with deeply stained chromatin with apparent large densely stained chromocenters. Nucleoli are small and multiple and most apparent in the nucleus in lower right. The cytoplasmic vacuolation usually appears beneath the cytoplasmic membrane. Wright-Giemsa stain.

humans, almost all L1 and L2 ALL have the terminal deoxynucleotidyl transferase (TdT) marker typical of cells of primitive differentiation and as an indication of maturation. This marker is absent in L3 ALL.

In human cases the L3 type ALLs are felt to respond poorly to therapy when compared to L1 and L2 ALL. Acute lymphoblastic leukemia type distinction has not had sufficient attention in animals for comment to be made on biological differences in response to treatment.

MATURE (PERIPHERAL) B CELL NEOPLASMS

B Cell Chronic Lymphocytic Leukemias/Small Lymphocytic Lymphomas

Chronic lymphocytic leukemia (CLL) of B cell type is a clonal disease with primary involvement of the bone marrow. The disease is primarily one of accumulation of long-lived cells of low proliferative rate and not of excess proliferation.^{12,17,41,46} The neoplastic cells are small and uniform in appearance and are immunologically competent. Small B cell lymphocytic lymphoma (SLL) is a solid clonal neoplasm of low grade primarily involving lymph nodes, spleen, and all parenchymal organs that is morphologically indistinguishable from B cell CLL.

Pathologic Features

In CLL, lymphocytosis is present in blood (frequently >100,000/ μ L) and bone marrow. In cats, where the

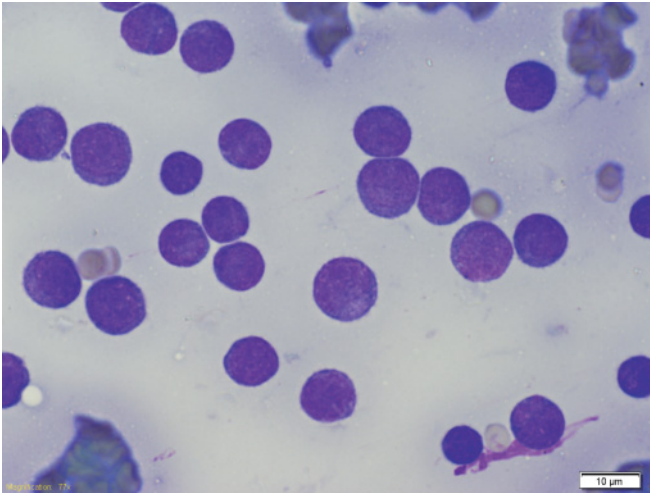


FIGURE 69.6 Blood smear from a cat with chronic lymphocytic leukemia. The leukocyte count was $>600,000/\mu\text{L}$ with the cat likely entering an accelerated phase of the disease. The smaller nuclei are more typical of the chronic disease with the larger nuclei part of a dividing population. The chromatin is quite densely stained with a narrow rim of quite basophilic cytoplasm. Nucleoli are not apparent in most nuclei. Wright-Giemsa stain.

disease appears most often, the cell type is of small cells with round nuclei typically 1 to 1.5 RBCs in diameter with a very narrow rim of cytoplasm. In dogs, the disease may be of similar cell type but, like humans, there is a second type with larger nuclei and much more abundant cytoplasm that is relatively lightly stained. Nucleoli are usually absent or very small and indistinct, and the chromatin is dense with little internal nuclear detail. Larger cells increase with progression of the disease (Fig. 69.6). These cells may have eosinophilic granules present indicating that they are large granular lymphocytes. Bone marrow is always heavily involved in CLL and there may be relatively solid involvement with almost complete phthisis of normal marrow cells. In early stages of CLL the bone marrow will be involved in a random fashion, unlike acute or chronic myeloid leukemias that tend to involve subendosteal areas first. Marrow is usually diagnostic with CLL and there is usually moderate involvement with SLL. In human CLL, with solid marrow involvement there are lighter stained areas that consist of slightly larger cells that have been recognized and described as proliferation centers. These consist of clusters of dividing cells and constitute part of the diagnostic criteria for CLL.

In cats with CLL, lymph nodes are rarely significantly enlarged and may be small or atrophic. In SLL, the nodes are enlarged with the capsule thinned and taut and the node has a diffuse architecture (Fig. 69.7). A remarkable finding in these nodes is numerous dilated lymphatics in what is likely a residual hilar area of the node that is distended with small lymphocytes of uniform type.

In CLL, the spleen is uniformly enlarged, primarily due to filling of the sinus areas with fading germinal centers arising from the arteriolar system, with an archi-

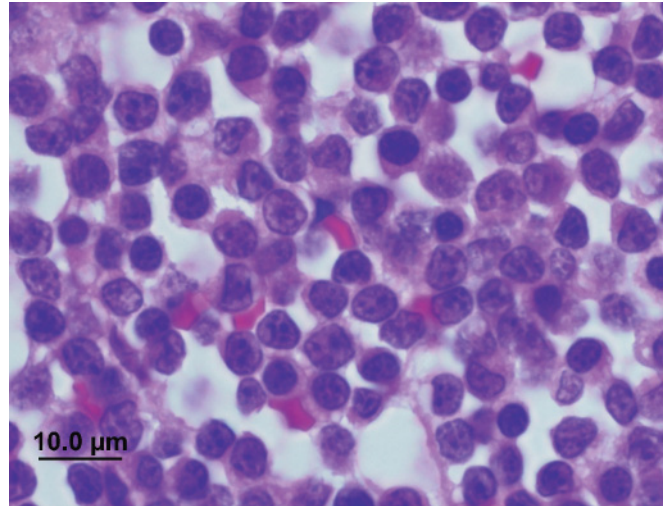


FIGURE 69.7 Lymph node from a cat with chronic lymphocytic leukemia. Note the small size of the neoplastic cells in comparison to surrounding RBCs. The small more dense cells would be more typical during chronic stages of the disease with the larger nuclei being the dividing population. The cytoplasm is minimal with moderate staining density. Mitoses are infrequently encountered. Hematoxylin & Eosin stain.

tectural presentation of a fine coalescing multifocal nodular proliferation.⁴⁷ There is frequently subendothelial involvement of the large muscular veins. In contrast small lymphocytic lymphoma tends to be multifocal and regional without uniform enlargement.

Chronic lymphocytic leukemia tends to involve most other tissues but most prominently the liver that presents with a periportal involvement. Other tissues involved include the choroid plexus of the brain, adrenal gland, and pancreas.

B cell CLL and SLL are uniformly positive with CD79a and CD1c and weakly positive for CD1a. They are negative for T cell markers.

The molecular specificities of CLL and SLL are largely undefined in animals, but probing of the immunoglobulin genes demonstrates clonality.⁴⁹ In humans, there is usually rearrangement of both the light and heavy immunoglobulin genes with some human cases of SLL also having rearrangement of the beta chain of the T cell receptor gene. About 10% of dogs with indolent B cell lymphomas have rearrangement of the T cell receptor gene.

The cell of origin for B cell CLL and SLL in animals is unknown. In humans, there is a lack of hypermutation of the variable portion of the immunoglobulin gene in both SLL and CLL that suggests that the origin of the tumor is a population of naive pre-germinal center B cells.³⁹

Chronic lymphocytic leukemia and SLL need to be differentiated from benign reactive hyperplasia because the cells lack atypia and individually cannot be recognized as neoplastic. The degree of increase in cell numbers in tissue or blood and the homogeneity of proliferation with cells of small cell type are the most important criteria in defining a neoplastic condition.

The diagnosis of B cell CLL is made on finding a leukemic blood picture with a very high number of circulating lymphocytes that consist of small mature-appearing cells with a very low mitotic rate. If the diagnosis is in doubt, the safest route is to retest to prove that there is a sustained marked lymphocytosis that can then be reliably assumed to be clonal. If the blood lymphocyte count is sustained above a level of about 10,000/ μ L, a bone marrow biopsy is indicated. Heavy infiltration of bone marrow with small cells of similar type confirms the diagnosis. In terms of specific diagnosis it is important to determine phenotype because T cell-type small cell lymphomas are more common than B cell-type at least in the cat.

The diagnosis of SLL is based on irregular enlargement of lymph nodes with focal lesions in both the liver and spleen and all affected areas being involved with an infiltration of very small mature-appearing lymphocytes. The blood picture may appear relatively normal in SLL in early stages.

Clinical Features

Chronic lymphocytic leukemia and SLL are slowly progressive clonal neoplasms of mature small lymphocytes: CLL begins in bone marrow and SLL begins in peripheral tissues. SLL is usually T cell type and is recognized most frequently in cats, less often in dogs, and infrequently in cattle and horses. It is seen in mature animals 5–10 years of age and in dogs 10–15 years. The disease may occur in younger animals but seldom in animals under 5 years old.

The accumulation of small cells of CLL may cause few clinical signs and is most often diagnosed incidentally in animals undergoing routine blood examination. Chronic lymphocytic leukemias and SLL affect all breeds of dogs and cats. In a review of 600 lymphoid neoplasms in cats, 23 cases (3.8%) were felt to be CLL- or SLL-type with these related entities present in about even proportions. In dairy cattle, clinical signs may include reduced feed intake and milk production, lethargy, loss of appetite, and melena if there is an ulcerated gastric tumor. Affected animals are usually thin and have hepatosplenomegaly.

Staging has not been applied in animals with CLL, but the Binet system of human medicine is straightforward. This system defines the stage of disease progression once the diagnosis is made. The human stages of progression can be roughly transposed in this manner:

1. absence of anemia or thrombocytopenia, tumor present in fewer than three lymphoid areas
2. absence of anemia or thrombocytopenia and involvement of more than three lymphoid areas
3. presence of anemia with less than 10 g/dL of hemoglobin and the presence of thrombocytopenia as defined by levels of less than 100,000/ μ L.

In animals this process involves comparing the current blood picture and topographic distribution of tumor with previous examinations based on the duration between examinations and rate of progression. It should

be remembered that if chemotherapeutic treatment is given, this will cause changes in cell type usually due to smaller cells that may mask the presence of conversion to an accelerated phase where there will be at least focal areas consisting of larger cells.

The survival of animals with SLL is largely undefined because the animals may have had the neoplasm for several years before diagnosis. When animals are presented in an accelerated phase, the tumor tends to be more responsive to treatment than when in a more indolent stage of disease. In general it is considered best to treat animals conservatively if they are not showing clinical signs. Treatment of non-clinical human cases of CLL may induce immune hemolytic anemia.

B Cell Polymorphocytic Leukemias

Polymorphocytic leukemia (PLL) is a clonal neoplasm involving the bone marrow and is characterized by a very high level of leukemic cells at presentation with marked splenomegaly, characteristic cellular morphology, and chronic course.^{23,32} The disease in animals is largely based on cell type without definition of the clinical entity. PLL can be of B cell- or T cell-type and in humans the ratio is about 80% B cell and 20% T cell.

Clinical Features

Polymorphocytic leukemia occurs in cattle and rarely in sheep and goats, and is seen most frequently in dogs and cats where it is usually considered to be chronic lymphocytic leukemia that has progressed to an accelerated phase.^{47,48} In humans, the disease has a well-defined presentation in elderly males that present with high levels of leukemic cells and marked splenomegaly.

Most cases described occur in older animals that are presented in poor condition with weight loss and marked splenomegaly. Lymph node involvement is seen. Typically the liver is not grossly enlarged but has histological involvement on a lobular basis.

Pathologic Features

Like CLL there is marked leukocytosis in excess of 50,000/ μ L typically present in the blood. The cells have a “hand mirror” shape and represent a stage of differentiation that is seen in both B and T cell types of PLL. The nuclei are round and the size of 2.0 RBCs with a moderate envelope of lightly stained cytoplasm. The chromatin has multiple (15–20) large densely stained chromocenters that are about 2 μ m in diameter and are outlined by clear areas of parachromatin joined by short, thin chromatin bands. Most cells lack nucleoli. There are occasional cells present that have slightly larger nuclei with less well-defined chromocenters that have nucleoli, and are considered to be the dividing population (Fig. 69.8).

The pattern of involvement in lymph nodes is typical of the B cell infiltration in CLL. In human nodes, the

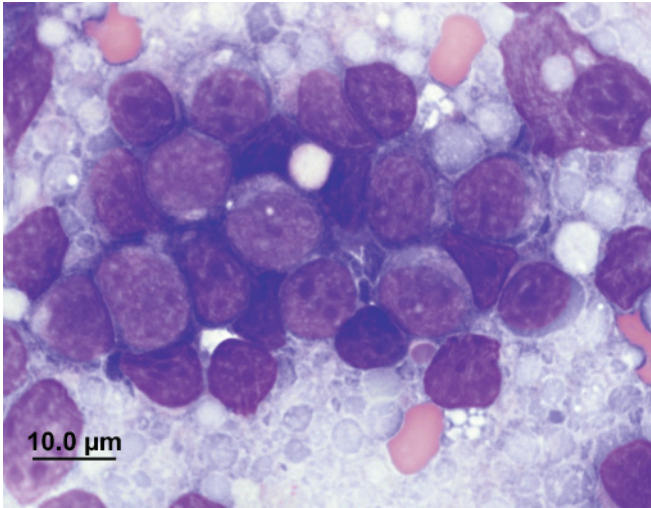


FIGURE 69.8 Lymph node aspirate from a dog with prolymphocytic leukemia. The nuclei are of intermediate size (1.5 RBCs in diameter). The chromatin pattern is characteristic with large chromocenters that are joined by broad chromatin bands with irregular parachromatin clearing that tends to give the nuclei a “spotted” nuclear pattern. Nucleoli are only visible in the larger nuclei and the cytoplasm is moderate in volume and staining density. Wright-Giemsa stain.

early disease begins around fading germinal centers like mantle cell lymphoma with gradual involvement of the paracortex. Involvement of the spleen is present with a fine multinodular pattern related to the arteriolar sheath vessels.

B cell type PLL is positive for CD79a and probably for CD20 and negative for CD3. There is clonal rearrangement of the immunoglobulin gene.

The tumor is derived from a relatively mature progenitor of either B cell or T cell lineage. Because this appears to be a stage of morphologic differentiation, the normal benign counterpart probably passes through this stage without ever forming a recognizable tissue mass.

Prolymphocytic leukemia must be differentiated from CLL that may have some larger sized cells in blood or tissue, but the bulk of the tumor population will have small compact nuclei with uniformly dense chromatin. Leukemic phases of mantle cell lymphoma may appear similar but nucleoli differ in most cells.

Lymphoplasmacytic Lymphoma

Lymphoplasmacytic lymphoma (LPL) is a clonal neoplasm of intermediate size cells that have nuclei similar to those of CLL and SLL.¹⁹ The cell type varies in having a greater cytoplasmic volume that is deeply amphophilic and gives the tissues a deep reddish tincture on architectural examination. The disease is otherwise similar to SLL with most nuclei lacking nucleoli and having a low mitotic rate (Fig. 69.9).

Lymphoplasmacytic lymphoma occurs in all domestic animals but is seen most frequently in older cats and horses.^{38,40,48,49} It occurs in the intestines of horses. About

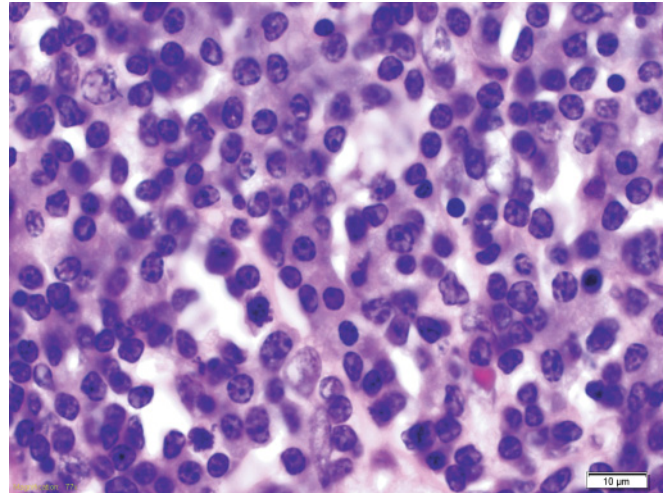


FIGURE 69.9 Kidney from cat with lymphoplasmacytic lymphoma. Histologically the lesion resembles chronic lymphocytic leukemia but with greater cytoplasmic volume that is more deeply amphophilic. Note the size of the nuclei in comparison to the RBC in the lower right. The cytoplasmic volume accounts for the wider spacing of the nuclei. Mitoses are infrequently encountered and cell boundaries are irregularly distinct. Wright-Giemsa stain.

half of the cases in the cat are also intestinal and some are mediastinal. The disease tends to be indolent.²⁸

Pathologic Features

Lymphoplasmacytic lymphoma does not usually have a leukemic phase; however, hyperglobulinemia may be present. Most cases of LPL have both surface and cytoplasmic IgM, are positive with CD79a and CD20, and are negative for T cell markers. However, it is possible for cells to have plasmacytoid morphology and stain for T cell markers.

The diagnosis of lymphoplasmacytic lymphoma is made on the recognition of the specific cell types in lymph node, spleen, or bone marrow. The cytoplasmic volume and deep staining density are the unique features of cells from animals with this disease.

Marginal Zone Lymphoma

Marginal zone lymphoma (MZL) is a B cell lymphoma of distinctive architecture and cytologic features that develops in a concentric layer of proliferation outside the mantle cell cuff of germinal centers (see Chapter 52). In human pathology, cells of marginal zone-type are referred to as “monocytoid B cells.”^{3,6}

Marginal zone lymphomas probably occur in all animals but are recognized most frequently in mature dogs and less frequently in cats.^{10,13,43} Marginal zone lymphoma appears to constitute approximately 15% of lymphomas in dogs and about 11% of non-Hodgkin’s lymphomas in humans. In human pathology, where MZL is divided into three disease presentations, the extranodal MALT-type lymphoma constitutes about

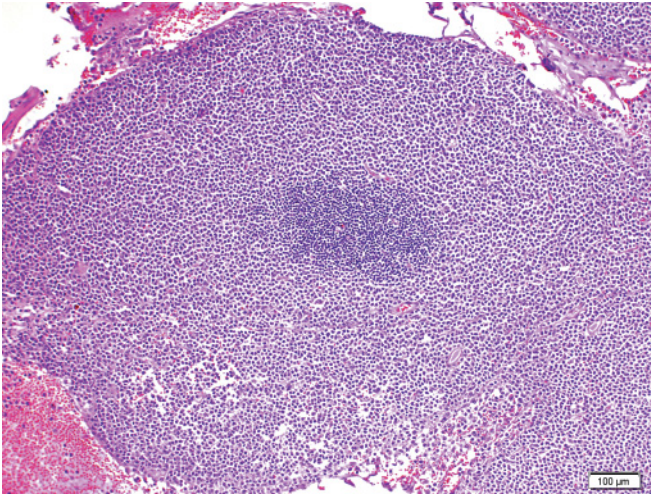


FIGURE 69.10 Lymph node from a dog with marginal zone lymphoma. The node has well advanced marginal zone lymphoma in which there is atrophy of surrounding paracortex with sinus ectasia. The small densely stained cells in the center are benign mantle cells that have collapsed into the dendritic bed of a fading germinal center. The surrounding cells are a malignant population of marginal zone cells. Note the lack of tingible body macrophages. Wright-Giemsa stain.

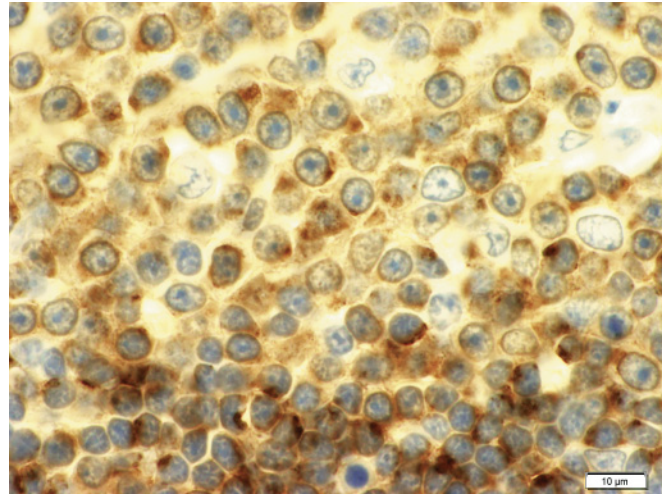


FIGURE 69.11 Lymph node from a dog with marginal zone lymphoma; CD79a stain. Cells at bottom of the lesion are mantle cuff cells at the edge of a fading germinal center. The marginal zone nuclei are 1.5 RBCs in diameter with a prominent single central nucleolus and with irregularly peripheralized chromatin with marked parachromatin clearing. The cytoplasm is relatively abundant which results in wider spacing of nuclei. Wright-Giemsa stain.

8% of all cases of non-Hodgkin's lymphoma, while the nodal-type is about 1.8%, and the splenic-type about 1% of total cases. A major species difference is that the disease always has diffuse involvement in humans and virtually always has focal and multinodular involvement in dogs.

Clinical Presentation

Marginal zone lymphoma occurs in mature large breed dogs, most frequently in submandibular lymph node, with only one site involved. The animals almost invariably feel well with normal appetite and activity. Nodes are always fully mobile. The second most frequent site in the dogs is the spleen with spread to hilar lymph nodes. Dogs eventually develop generalized lymphadenopathy but continue to feel well.

Pathologic Features

Leukemic manifestations of MZL have not been identified in animals. Bone marrow is seldom involved in MZL. The capsule of affected lymph nodes is thin and taut with the peripheral sinus preserved. Architecturally there is follicular hyperplasia with germinal centers extending into medullary areas. Usually there is some degree of medullary sclerosis indicating a long period of hyperplasia. In early stages, some germinal centers may still retain antigen-related polarity but with progression there is collapse of germinal centers with only a central area of mantle cells remaining. Marginal zones then appear as a lighter staining area of cells surrounding these dense clusters of smaller mantle cells (Fig. 69.10).

The splenic involvement invariably is multifocal and locally extensive. The distribution of tumor foci of MZL is related to splenic end arterioles that dictate a regular periodicity of nodular distribution. These nodules then irregularly coalesce with disease progression (Fig. 69. 11). Splenic MZL is usually accompanied by internodular areas of plasmacytosis often with atypical features and accompanied by focal areas of sclerosis that are usually identified as "fibrohistiocytic nodules." Because progression of MZL is slow, it is likely that many cases have been identified as areas of nodular hyperplasia. In general, when nodules are small and discrete, clonality studies tend to indicate that the condition is benign. Alternatively, when nodules are large and multifocal clonality studies usually indicate malignancy.

Marginal zone lymphomas in animals are strongly positive for B cell markers including CD79a and CD20, and are negative for T cell markers (Fig. 69. 12). Consistent cytogenetic and molecular changes in MZL have not been identified in animals. In humans, the most consistent features of this type are seen in the splenic form in which trisomy 3 and trisomy X and the deletion of (q22q24) have been reported in a small group of patients.

The cell of origin in MZL is not known in dogs. In humans, the close association between chronic immune stimulation caused by *Helicobacter* sp. gastritis and MALT lymphoma strongly suggests an origin from a post-germinal center B cell.

In all tissue areas the key to diagnosis of MZL is based on the recognition of cellular proliferation arising outside the mantle cell layer. In advanced cases, most areas will consist entirely of marginal zone cells but a few areas may be identified with fading marginal zone

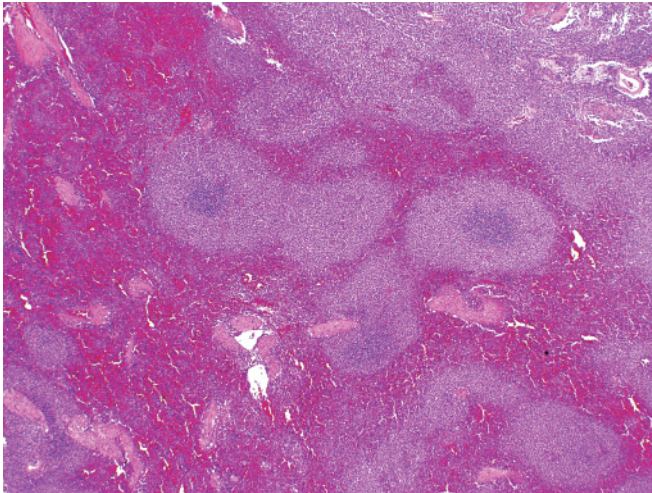


FIGURE 69.12 Spleen from a dog with marginal zone lymphoma. There are irregularly coalescing areas in the center and a large area of diffuse proliferation in the upper right. Note the atrophy of periarteriolar lymphoid sheaths. Wright-Giemsa stain.

cells. Marginal zone cells have nuclei 1.5–2 RBCs in diameter with moderately hyperchromatic chromatin that tends to be partially peripheralized onto the nuclear membrane with mild irregular parachromatin clearing. The other consistent finding is a prominent single central nucleolus. In addition, MZL tends to have relatively abundant and lightly stained cytoplasm. This combination of vesicular nuclei and abundant cytoplasm gives marginal zone areas a lighter staining aspect on architectural examination. A consistent cytologic characteristic of early MZL is that mitoses will be absent in most 400× fields. With progression in the dog (i.e. after 18 months to 2 years), there will be 3–4 mitoses per 400× field with an equivalent number of tingible body macrophages.

In lymph nodes, MZL needs to be differentiated from T cell lymphoma that may look similar at the architectural level because of the appearance of fading germinal centers. These centers are peripheralized in T cell lymphoma and in MZL the proliferating cells are surrounding the fading centers. Both types of lymphoma share a low mitotic rate. Differentiation is made on the basis of the T cell lymphomas tending to have smaller nuclei with more compact chromatin and less cytoplasm. Immunophenotyping is useful in differentiating these two types of lymphoma.

Clinical Features

Marginal zone lymphoma is generally not associated with clinical signs, but with current usage of ultrasound, splenic masses are readily identified. Because the most common form of focal splenic mass in the dog is hemangiosarcoma, splenectomy is the most frequent approach to treatment. Almost invariably MZL has not spread to other sites at the time of splenectomy.

In contrast, MZL in lymph nodes in the dog tends to be diagnosed in more advanced cases, generally at stage

2a or 3a. In nodes that are completely effaced by MZL, the nodular nature of the disease may not be apparent until immunophenotyping is done. Most cases of nodal MZL in dogs involve peripheral nodes but occasionally internal nodes may be involved.

Marginal zone lymphoma can progress from focal to multifocal to generalized over time. At the architectural level, the lesion progresses from multinodular to diffuse and the cytologic changes include appearance of low numbers of mitotic figures and an accompanying presence of tingible body macrophages. MZL tends to respond well to chemotherapy at all stages of disease but may respond better as the number of mitotic figures increases.

Animals with a prior diagnosis of MZL tend to remain in phase 3a or 4a and tend not to become leukemic. Animals in advanced stages tend to retain normal appetite and activity and all nodes appear to be equally diagnostic for sampling purposes.

A major diagnostic concern with MZL is that the large nucleoli in intermediate-sized cells are cytologically interpreted as high-grade lymphoma. MZL can be accurately diagnosed if a 2mm diameter Tru-cut biopsy needle is used in most tissues except liver and kidney.

Some cases of canine MZL will continue to respond to the same treatment protocol with prolonged remissions over a period of 1–2 years. An indication of the very slow spread of MZL is indicated by three cases treated only by splenectomy that did not have subsequent recurrence in other tissues. In humans, 5-year survival with the splenic form of MZL is 65%.

Mantle Cell Lymphoma

Mantle cell lymphoma (MCL) is a distinct subtype of lymphoma in humans and animals that is characterized by architectural origin around fading germinal centers with nuclei characteristically uniformly round and 1.5 RBCs in diameter with dense chromatin with little internal detail.^{24,30,51} Most nuclei lack visible nucleoli and the tumor has a low mitotic rate. Mantle cell lymphoma has been recently recognized in human pathology and is identified as a distinct entity arising from a translocation T(11;14) and by over expression of cyclin D1 protein.

Mantle cell lymphoma is recognized in cats and dogs and probably occurs in all mammals. Mantle cell lymphoma constitutes 5% of human lymphoma in North America and twice that level in Europe with a 3:1 male predominance. In humans, the disease occurs in elderly patients. Eleven cases of canine MCL were identified in a collection of 461 cases with an incidence in the dog of about 2%. The age ranged from 1.5 to 15 years with a mean of just under 8 years. In a comparable period, since the diagnosis began to be recognized in 1992, one case has been recognized in 150 cases of lymphoma in cats.

Like MZL, MCL is much more likely to occur in the spleen in the dog. Mantle cell lymphoma tends to present as a multinodular and multifollicular prolifera-

tion that then undergoes coalescence. Associated changes in spleen include atypical plasmacytosis with fibrohistiocytic nodules and occasionally focal hemorrhagic infarction.

In both humans and dogs, a “blastoid variant” of MCL occurs that is identified by slightly larger nuclei with consistent small nucleoli. This form tends to become leukemic and develop bone marrow involvement. In the dog, this type of presentation has a characteristic diffuse multinodular architecture. The nodules enlarge in size and in so doing appear to compress their own blood supply undergoing ischemic degeneration followed by hemorrhage.

Pathologic Features

In dogs, the blastoid-type of MCL will present with febrile disease accompanied by anemia and gammopathy that is reversed on splenectomy.⁵ In humans, a variable pattern is seen that tends to be paratrabeular with progression to a diffuse pattern. Lymph node MCL in the dog is generally identified in animals with generalized lymphadenopathy. Typically the nodes are markedly enlarged without involvement of perinodal tissues. Architecturally the nodes have a diffuse architecture with loss of germinal centers and proliferation compressing medullary structures generally without medullary sclerosis (Fig. 69.13). The follicular nature of MCL may only be evident on phenotypic staining.

Mantle cell lymphoma is more difficult to recognize histologically. The architectural identity becomes dependent on identifying a few slightly smaller more densely stained benign mantle cells at the center of rounded areas of cellular proliferation of slightly larger

cells. Cytologically, MCL has round nuclei that are densely stained and generally lack nucleoli. The cells are of the same size or slightly larger than those of T-zone lymphoma and lack nuclear indentations. Nucleoli are small or inapparent and cells have a relatively wide rim of cytoplasm giving the nuclei a neatly separated appearance similar to T-zone lymphoma. Like other indolent lymphomas, the mitotic rate is low and none may be found in a 400× field with the small type of mantle cell lymphoma, whereas 3–4 per 400× field can be found with the blastoid-type of MCL. Typically tingible body macrophages are not present.

In dog and cat spleen, MCL is characterized by multifocal proliferation and is locally extensive (Fig. 69.14). With the small cell-type of MCL there will be progressive coalescence of splenic nodules and internodular areas of atypical plasmacytosis and fibrosis as occurs with marginal zone lymphoma. As noted earlier, the blastoid variant of MZL tends to involve the spleen uniformly in the dog and has not been identified in the cat. Cytologically the blastoid-type of MCL has nuclei that are 1.5 RBCs in diameter with much more variation in shape than with MZL. The chromatin pattern is hyperchromatic and coarse granular with irregular parachromatin clearing and each nucleus having 1–2 small nucleolus that are much smaller than the large single nucleolus of MZL. In splenic blastoid-type MCL in the dog there is typically subendothelial colonization of larger venous sinuses. Other tissues that are typically involved include lymph node, bone marrow, and liver. The liver involvement is by portal cuffing and occasionally implantation eccentric to central veins.

MCL is uniformly positive with the B cell markers CD79a and CD20, and negative with CD3 and other

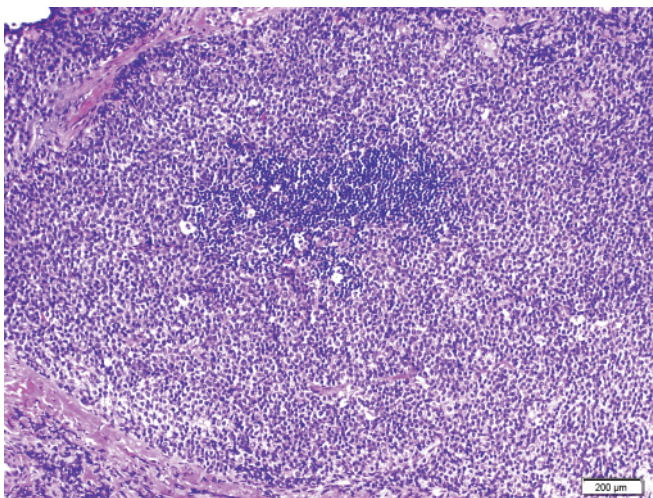


FIGURE 69.13 Lymph node from a dog with mantle cell lymphoma. Architecturally the pattern resembles marginal zone lymphoma with the proliferating cells surrounding a fading germinal center. The proliferation of cells around the mantle cell layer rather than eccentric to it identifies the neoplastic cells as B cells. Identification of the cells as small to intermediate nuclear type with small nucleoli is essential to recognize mantle cell lymphoma. Wright-Giemsa stain.

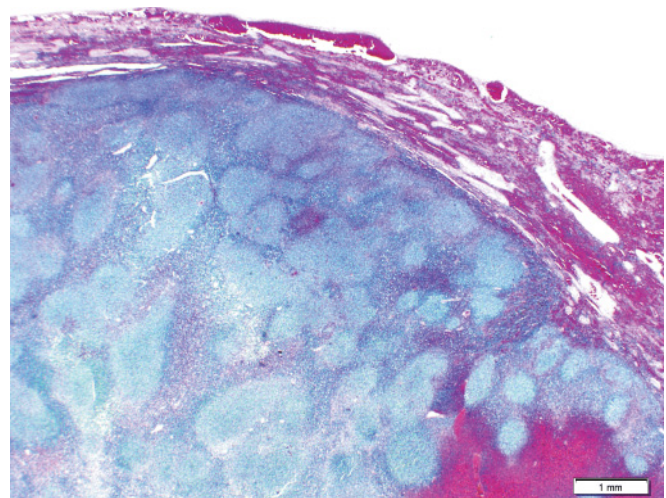


FIGURE 69.14 Spleen from a dog with mantle cell lymphoma. A cohesive nodule of coalescing foci is a typical presentation that is frequently accompanied by focal areas of hemorrhagic infarction as is apparent in lower right. The proliferating areas are lightly and irregularly outlined by more deeply stained cells consisting mostly of small benign plasma cells. Note the atrophy of periaarteriolar lymphoid sheaths in surrounding more normal spleen (above and to the right).

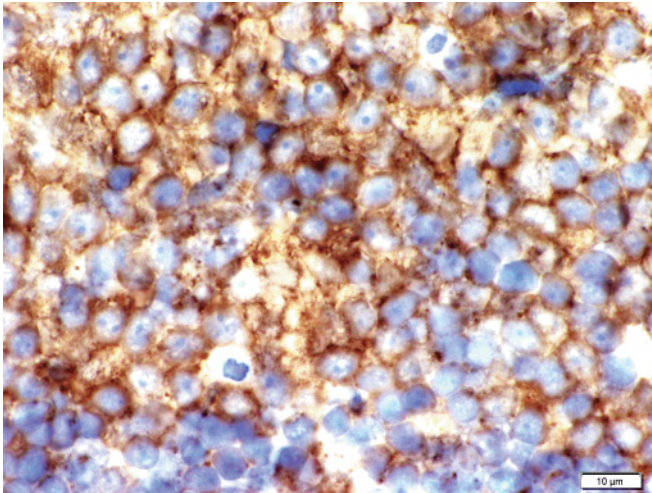


FIGURE 69.15 Lymph node from a dog with mantle cell lymphoma; CD79a stain. The closely aggregated nuclei at the bottom are the edge of the fading mantle cell cuff evident at lower power in Figure 69.13. Note that the major difference between the benign and malignant mantle cells is only a slight enlargement of nuclei with the presence of prominent small nucleoli, but with more abundant cytoplasm that is deeply stained with CD79a. While the nucleoli are apparent and occasionally even two are present, they are much smaller than those typical of marginal zone lymphoma and the nuclei themselves are smaller.

T-cell markers (Fig. 69.15). In dogs, MCL tends to stain lightly and uniformly for lambda light chain.

Nothing is known about genetic changes characteristic of MCL in animals. In human MCL, translocation (t(11;t14)(q13;q32) is usually present and is considered presumptive for diagnosis of MCL. In human cases, the oncogene dysregulated by the translocation has been termed *CCND1* and encodes for the cyclin D1 protein that is over expressed in tissues.

The cell of origin in animals is not known. In humans, it is hypothesized that MCL is derived from naive marrow B cells that are pre-germinal center and migrate from the marrow to the mantle cell area.

Mantle cell lymphoma is a type of lymphoid neoplasm with a nodular gross pattern in all tissues that is initially focal and becomes coalescent as the disease progresses. In early cases, apparent origin from germinal centers is evident by fading follicles within some foci of proliferation. An additional useful finding is the presence of areas of protein insudation that are present as follicular hyalinosis in a germinal center that has receded with the protein remaining.

Mantle cell lymphoma must be differentiated from other indolent lymphomas that have a nodular architecture including follicular lymphoma and MZL. MCL might be confused with follicular lymphoma grade I where the concentric sites tend to have nuclei that are more irregular in shape than those of MCLs. MCL is differentiated from MZL because of smaller nuclei without the prominent single central nucleolus characteristic of MZL. In advanced cases MCL needs to be differentiated from chronic lymphocytic leukemia.

Clinical Features

Mantle cell lymphoma in animals presents at advanced stages of 3–4 and staging is based on recognizing the number of areas of nodal involvement of both internal and peripheral nodes as well as hepatosplenomegaly. On fine needle aspirate, the cells are slightly larger than those of T-zone lymphoma and lack the shallow nuclear indentations. In advanced cases, tumor is present in the liver.

Little is known about the progression of MCL in animals. In humans, the most significant prognostic factor is shortened remission time after treatment preceding recurrence. Poor prognostic signs in humans are old age, poor performance at the time of diagnosis, advanced stage of the disease, increased lactate dehydrogenase levels, splenomegaly, and anemia. Other symptoms, consisting of fever, night sweats, and loss of body mass, are important negative factors in humans as well as for the appearance of leukemia.

Nothing is known about treatment or survival in animals with MCL. In human cases of MCL, survival is 3 years with an absence of long-term survivors and survival with the blastoid variant of MCL is generally less than 2 years. This seems to be at variance with very limited experience in dogs where splenectomy of dogs with blastoid-type MCL has been followed by long-term survival.

Follicular Lymphoma

Follicular lymphoma (FL) is a type of B cell neoplasm composed of centrocytes and centroblasts with at least partial follicular architecture.^{14,20,42} A major distinguishing feature of follicular lymphoma is that the cell composition of each follicle must be exactly similar to that in other follicles. This serves to distinguish true FL from various stages of follicular hyperplasia.

True FL in animals is an uncommon diagnosis. In a collection of 502 cases of canine lymphoma, there were 20 cases of follicular lymphoma and 15 cases of benign and atypical follicular hyperplasia. The true incidence of the disease in dogs is likely somewhere between 2% and 4% of total lymphoma cases. In one review of 176 cases from the New York City area, there were eight cases of FL-type 3, two cases of FL-type 2, and none of FL-type 1 for a total incidence of 5.7%. Increased incidence in that population may have been because these cases had routine immunophenotyping carried out assisting recognition of advanced cases. In a major review of the WHO classification system of human lymphomas, it was found that architecture alone was adequate for diagnosis of FL. In a review of 602 cases of feline lymphoma by histopathology alone, there were six cases (1%) of FL. A review of 1198 cases of bovine lymphoma, identified by inspection at Canadian slaughter plants, identified four cases (0.3%) of FL.⁵⁰ Follicular lymphoma appears to be rare in the horse, where the incidence appears to be less than 1%.²⁷ In humans in North America and Western Europe, 25–40% of lymphoma cases are FL. Follicular lymphoma is infrequent

in developing countries: it is likely that there are local influences involved. In support of that concept, a review of 238 cases of canine lymphoma from Ohio, in which immunophenotyping was carried out, found one case of FL.

Clinical Presentation

Affected dogs vary between 2 years and greater than 10 years of age.⁴⁵ Animals with FL tend to have both peripheral and internal lymph node involvement. These nodes tend not to be enlarged, suggesting early spread and slow progression. In the dog, the nodes most likely involved are those around the head and neck, including prescapular and popliteal areas as well as mediastinal and sublumbar nodes. The nodes are always non-painful and they are not fixed to the skin or deeper structures. Follicular lymphoma, like chronic lymphocytic leukemia, is usually found on incidental examination for dental care or other routine examination. With extensive lymph node involvement there is usually at least focal involvement of liver and multifocal involvement of spleen. Bone marrow may be involved but it is not apparent on examination of blood.

Pathologic Features

Virtually nothing is known about the presence of cells in blood of animals with follicular lymphoma. In humans, neoplastic cells are seen in blood in about 30% of cases. These neoplastic cells resemble those seen in nodes of dogs with T-zone lymphoma and have very sharp shallow nuclear indentations.

In the few cases of FL examined in which bone marrow was involved, nonspecific changes of cancer were present including an increased coarse hemosiderin and plasmacytosis. The follicular pattern is maintained when FL involves the bone marrow and the tumor tends to have a paratrabeular distribution.

In FL, the disease usually occupies an entire lymph node, but rarely nodes have partial involvement.⁴⁸ The pattern consists of follicles that are tightly faceted (Fig. 69.16). A characteristic of FL is that there is complete absence of mantle cell cuffs and internodal areas tend to consist of the post-capillary venules and small arterioles with increased numbers of RBCs and a variable complement of small, medium, and large lymphocytes (Fig. 69.17). The peripheral capsule is characteristically thin and taut, with the peripheral sinus compressed and compromised in areas where there is colonization of extranodal tissues.

Cytologically most cases in the dog are recognized at a grade II level when there is a mixture of centrocytes being the smaller cells with small or inapparent nucleoli and centroblasts that are the large more rapidly dividing population with multiple usually peripheralized nucleoli. The same proportion of centrocytes to centroblasts will be present in each nodule: that is the primary distinction between FL and follicular hyperplasia (Fig. 69.16 and Fig. 69.17). In addition, whereas tingible body macrophages are a characteristic of benign follicular

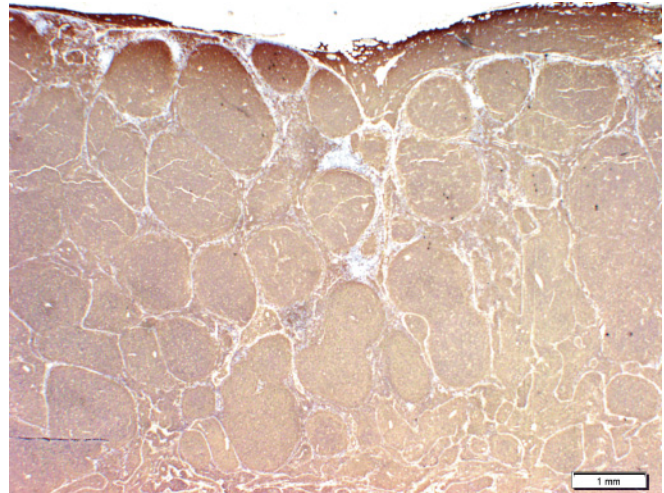


FIGURE 69.16 Lymph node from a dog with grade III follicular lymphoma; CD79a stain. With hematoxylin and eosin staining the definition of the follicles is lost and the node largely appears to have a diffuse proliferation except for follicles delineated near the node capsule. Note that in the upper right there is diffuse lymphoma proliferating outside of the node capsule with focal areas of diffuse progression acceptable in grade 3 lesions. A characteristic of follicular lymphoma, that distinguishes it from hyperplasia at the architectural level, is the complete absence of mantle cell cuffs. Note that follicular lymphoma cells of similar type extend right to the edge of each follicle. Detailed examination of the node reveals that small arterioles and post-capillary venules are all located between the nodules and never within them.

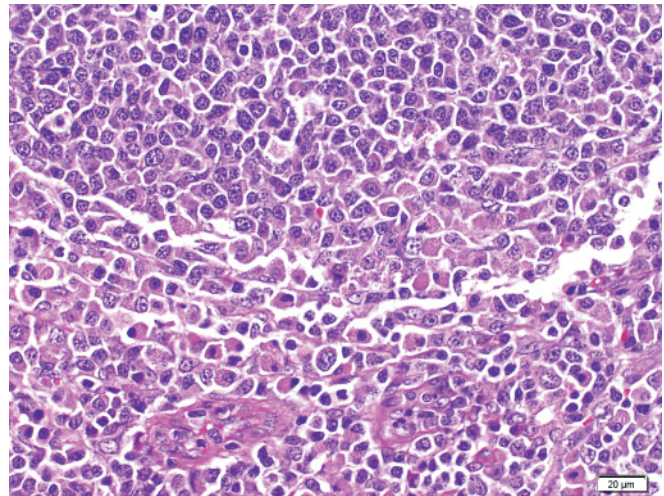


FIGURE 69.17 Lymph node from a dog with grade II follicular lymphoma. The cells in the upper half of the image are primarily centroblastic B cells of neoplastic follicles. A light stroma separates the follicle from the intrafollicular area below. There are two post-capillary venules in the internodal tissue that have heavy transmural cellular traffic. Note the variation in cell type in the interfollicular areas that includes plasma cells as well as small, medium, and large lymphocytes. Wright-Giemsa stain.

hyperplasia, they tend to be rare or absent in FL. Occasionally in areas where tingible body macrophages are still present these may be identified as areas of fading follicles.

The WHO system of classifying FLs is a system of grading the proportion of centroblasts in each field. Basically, a level of 0–5 centroblasts per high power field (400×) is considered grade I, with 6–15 per high power field grade II, and more than 15 centroblasts per high power field is grade III. There is quite wide variation in grading between individuals. Because these diseases are so rare in animals, little effort has been made to count specific cell types other than to note that there is a general mixture present without centroblasts predominating.

Follicular lymphoma in the spleen is largely undescribed in animals. In humans, FL tends to symmetrically involve the entire spleen. On the basis of the few cases described in animals, involvement of the spleen in FL is characteristically diffuse with involvement on the entire body of the spleen. In cat and mouse, the sinus areas are spared with the focal proliferations quite sharply defined. Follicular lymphoma in the spleen is characterized by complete loss of mantle cell cuffs and, as in lymph nodes, there is the same distribution of cell types in all regions of each follicle.

The other organ most consistently involved is the liver. Follicular lymphoma in the liver tends to exclude areas of hematopoiesis from portal areas and similar changes are present in marrow with the follicles tending to be identified on low power examination by complete phthysis of fat cells and megakaryocytes.

Follicular lymphomas are strongly positive with CD79a and CD20 and are negative with CD3. In most cases of animal FL, cytoplasmic immunoglobulin can be identified by immune staining and most can be shown to stain positively with lambda light chain and negative with kappa light chain, which is also a useful means of identifying clonality.

It is well established in human pathology that FL is a result of neoplastic transformation of a post-germinal center cell. No specific characteristics have been identified for FLs in animals. In humans with FL, the heavy and light immunoglobulin chains are rearranged with the variable regions and there are extensive somatic mutations. In addition, there is extensive intraclonal diversity, indicating that mutations are ongoing similar to those that occur in benign germinal centers. In human FL, characteristic genetic alterations are chromosomal translocations of the long arms of chromosomes 14 and 18 with t(14;18)(q32;q21). A co-migrated segment that is transcribed is called BCL2 and this gene is over expressed in FL so providing a survival advantage by preventing the normal process of turning off the anti-apoptosis gene.

The major differential diagnosis for FL is benign or atypical follicular hyperplasia. In benign follicular hyperplasia there is usually some level of mantle cell cuff remaining, even in chronic follicular hyperplasia. A further distinction in follicular hyperplasia is that many of the follicles within the tissue have antigen-related polarity that signals that these structures are responding normally to antigen stimulation. In follicular hyperplasia, the involvement of perinodal tissue is

frequently present but is almost never nodular or follicular as usually occurs in FL.

Clinical Features

Animals with FL, as is typical of all indolent lymphomas, retain normal appetite and activity and frequently present with advanced disease as a result of the owners noticing enlarged peripheral lymph nodes. Little is known about management of FL in animals, but the general management in humans appears logical. Animals having grades I–II lymphomas could be treated conservatively while those with grade III FL should be considered to have aggressive large B cell lymphoma and given multi-agent therapy.

Nothing is known of risk factors for survival of animals diagnosed with FL. Changes considered important in predicting survival in human patients with FL are age, time since diagnosis, presence of nodes larger than 3cm in diameter, and the number of extranodal sites involved. Negative factors accompanying these are the presence of B cell symptoms including night sweats and elevations of serum lactic dehydrogenase.

Little is known about specific treatment for FL in animals. In humans, the major management therapy for patients in stage I or II of the disease is radiation which may provide relapse-free survival for 10 years or more. Alternatively, alkylating agents, including chlorambucil and cyclophosphamide, provide remission rates of 30–60% in previously untreated human patients. Newer strategies of therapy include anti-B cell antibodies. The current median survival of human patients with FL is about 9 years and after relapse about 4.5 years. In dogs with FL it is likely they have had the disease for up to 2 years before diagnosis and have a good chance of living 2–3 years after recognition and treatment.

Diffuse Large B Cell Lymphomas

Diffuse large B cell lymphomas (DLBCL) are tumors of large transformed lymphocytes with nuclei at least twice the size of two small lymphocytes.^{2,18,29} The nuclei can vary markedly in shape and the cells generally have vesicular chromatin.^{21,22} The cytoplasmic volume and staining density vary with the subtypes. DLBCL are heterogeneous and have variable numbers of proliferating cells with wide variation in antigenicity, molecular and genetic features, and clinical behavior.^{4,9}

Diffuse large B cell lymphomas occur in all domestic animals, including birds, and are the largest and most frequent lymphoid neoplasms in most species including humans.^{33,44} In cats and cattle, DLBCL is associated with retroviral infection but lymphomas also occur in the absence of viral involvement. It is likely that in all species chronic benign lymphoid hyperplasia that occurs in association with a variety of chronic diseases may be a risk factor for malignant transformation of benign B cells.

A histological study of 1,198 lymphomas in cattle, without using immunohistochemistry, found that 366 were large cell-type and 424 were of large cleaved cell-type, together comprising 66% of the total cases. Characteristics of the population were not known, but because these were adult cattle sent for slaughter, most were assumed to be female and of dairy breed. In 136 cases of lymphomas in domestic pigs, 81 (60%) were of large cell-type and in 502 cases of canine lymphoma and leukemia there were 69 cases (14%) of large cell lymphoma including 19 cases of large cleaved cell-type. The median age of 47 of these dogs was 6.1 years and approximately equal numbers were male and female. Of 751 hematopoietic neoplasms of domestic cats, there were 314 (42%) cases of large cell lymphoma with 76 of these of large cleaved cell type, 184 cases of immunoblastic large cell lymphoma, and 115 cases of immunoblastic polymorphous type. In the total cat population, 58% of cats were male or neutered males with the remainder female or neutered females. The ages of cats with lymphoma ranged from 1 to 22 years. In 90 horses with lymphoid neoplasms there were 19 cases of diffuse large cell type and two cases of large cleaved cell type. In equines there was a slight majority of female cases, most of which were older than 10 years.

Pathologic Features

It is currently felt that neoplastic cells are always present in the blood with the conditions of spread determined by the intercellular adhesion molecules on the surface of malignant cells. In general, about 15% of cats with lymphoma are frankly leukemic with associated lymphocytosis. There is little prognostic value in identifying leukocytes in blood of an animal that has an established diagnosis of lymphoma and is not suffering any type of cytopenia. Examination of blood and marrow is an important and essential step in staging animals and predicting response to therapy.

It is easiest to recognize neoplastic cells in blood when such cells are large, and have nuclei with prominent nucleoli and uniformly finely distributed chromatin. That said, it is important to note that the neoplastic cells in blood frequently differ morphologically from the neoplastic population in marrow or in other tissues. Bone marrow is always involved in DLBCL if the disease has progressed for some time. The manner in which neoplastic cells involve the marrow may vary, including diffuse, paratrabeular, and multifocal interstitial. With focal heavy involvement of marrow there is usually exclusion of fat cells and phthisis of normal marrow lineages that is indicative of tumor formation. The advent of immunocytochemistry now permits the identification of minor populations of neoplastic cells in bone marrow that are not identified on routine examination.

A number of types of large B cell lymphomas are recognized in the recent WHO classification. Three of these are distinguished on the basis of their anatomic location including intravenous or intravascular, medi-

astinal, and primary effusion types of lymphoma. A new entity based cytology alone, is the plasmablastic-type that has general characteristics of plasma cells but with larger nuclei and nucleoli and a higher dividing fraction. Two subtypes of large B cell lymphoma are of mixed-type including the T cell-rich large B cell lymphoma and a closely related type known as lymphomatoid granulomatosis that tends to occur in the lung.

In general, lymph nodes enlarged in large B cell lymphomas have diffuse architecture, frequent bridging of the node capsule, and colonization of perinodal tissues. Large cell lymphomas tend to compress and destroy medullary structures and are not associated with medullary sclerosis as is usually found in the indolent lymphomas that appear to develop after a long period of benign hyperplasia. A common division of subtypes of large cell lymphomas is on the basis of nucleolar number and position. With this classification the immunoblastic lymphomas (IB) have a large single central nucleolus while the centroblastic (CB) types have multiple peripheral nucleoli (Figs. 69.18 and 69.19). These were recognized as distinct types in the working formulation with the assumption that the immunoblastic-type was of higher grade; however, little difference was found between these two in subsequent trials. Currently it seems reasonable to divide lymphomas cytologically on the basis of nucleolar type until it is possible to determine if this distinction infers a survival advantage in treated animals. In a study of 1,017 canine lymphomas, there were 150 cases of large cell lymphoma of CB-type

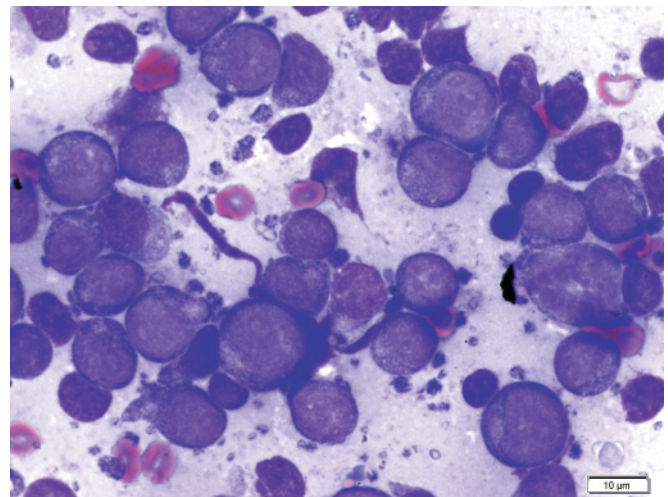


FIGURE 69.18 Lymph node aspirate from a dog with diffuse large B cell lymphoma; Wright-Giemsa stain. The nuclei are 1.5–2 RBCs in diameter with densely stained chromocenters that encircle multiple largely central nucleoli. The nuclei of intermediate size have a similar chromatin pattern with multiple nucleoli that suggest these are part of the same neoplastic population. There are a number of small (benign) lymphocytes present that have densely stained nuclei similar in diameter or slightly larger than RBCs. The cytoplasm is relatively abundant and highly basophilic. There is a cell in mitosis at the center right margin.

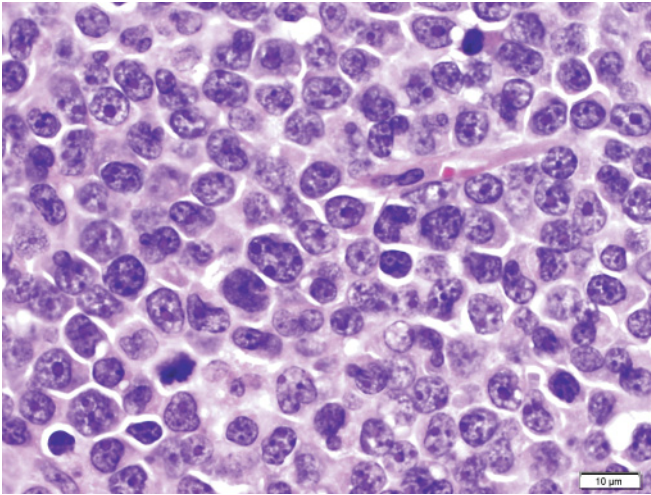


FIGURE 69.19 Lymph node from a dog with diffuse large B cell lymphoma. The large oval nucleus in the center with peripheralized nucleoli is a typical centroblastic nuclear type with mild parachromatin clearing. There is a mitotic cell in the lower left and an apoptotic cell in the upper right. There is more anisokaryosis in this field than is typically present with this subtype of lymphoma. Wright-Giemsa stain.

in which the mitotic rate was determined to be 12.13 per 400 \times field while in the same study 55 cases of IB-type had a mean mitotic rate of 8.7 mitoses per 400 \times field. These differences were statistically significant, suggesting that this distinction might relate to biological behavior.

Cytologically, both the immunoblastic and centroblastic cell types tends to have round to oval nuclei 2–2.5 RBCs in diameter and coarse granular or branched chromatin patterns with mild parachromatin clearing. The cytoplasm is almost always moderate in volume and staining density with cell boundaries irregularly distinct. There are usually about the same number of apoptotic nuclei and mitoses as is seen in Burkitt-type lymphoma but not as many tingible body macrophages.

The plasmablastic variant of large B cell lymphoma occurs in dogs, cats, and horses. The distribution of the tumor can vary widely and involve almost all tissues including lungs and skin. Major distinguishing features of this type of lymphoma are large nuclei (>2.0 RBC diameters), marked variation in chromatin distribution, and very abundant eccentrically placed and highly amphophilic cytoplasm. A major difference between plasmablastic lymphoma and plasmacytoma is the presence of nucleoli and a higher mitotic rate in the plasmablastic subtype. There are some differences in homing patterns with the plasmablastic-type of lymphomas tending to mimic plasma cells in homing to medullary cords where they may be mistaken for benign plasma cell expansion if not examined in detail.

The intravascular variant of large B cell lymphoma is rare. It may be found in any tissue but is most frequent in the central nervous system. Animals with this form of lymphoma usually present in good condition

with a rapid onset of nervous signs that begins with caudal paresis and rapidly ascends to convulsions and collapse with death occurring in 1–2 days after onset. Histologically, there is dilation of veins in the cerebral meninges and subdural vessels of the spinal cord. Oddly, the cells appear in clusters that distend vessels but do not appear to be attached to vessel walls; however, the tumor is not apparently leukemic. There is marked anisokaryosis with multinucleated and binucleated cells present. The nuclei are vesicular with peripheralized and branched chromatin patterns and multiple prominent nucleoli. The cytoplasm is moderate in volume and staining, and cell boundaries are generally distinct. Similar intravascular lymphomas may be found in the heart and lungs.

Mediastinal large B cell lymphomas are rarely encountered or described but occur in dogs very similarly to what is described in humans. These lymphomas also occur in the cat and perhaps other species. In humans, the disease occurs in young adults with a female to male ratio of 3:1. Specific identification of the neoplasm is important because mediastinal large B cell lymphoma rapidly invades surrounding tissues. This disease is usually identified by ultrasound-guided fine needle aspiration and Tru-cut biopsy. Cytologically, the cells vary markedly in size and shape and are supported by a dense fine fibrovascular network that surrounds cells down to groups of two or three. Binucleated forms are occasionally present.

The primary effusion-type large B cell lymphoma is rare in humans and has recently had a single report in animals. The disease is defined as an effusion of fluid containing neoplastic large B cells in serous cavities including the pleura, pericardium, or peritoneum in the absence of solid tumors in the underlying tissues. In humans, the disease is seen more frequently in immunosuppressed and post-transplant patients.

Involvement of the spleen in large B cell lymphoma is initially multifocal and rapidly becomes coalescing. The multifocal involvement may occur as 1–2 mm foci grossly termed “sago spleen”, or the tumor may be a single large isolated mass. Focal large cell splenic lymphoma is an entity in the horse and the tumor may obtain a mass of 11.3–15.9 kg (25–35 lb). Diffuse involvement of the spleen is prominent in cows with lymphoma and rupture of the capsule may be the first indication that the tumor is present with the cow becoming recumbent due to anemia. In general, the larger the tumor the more likely it is to be associated with hemorrhage and infarction. Large B cell lymphoma frequently also involves the liver and kidney, but almost any other tissue in the body may be affected including uterus, skeletal muscle, tonsil and rarely bone.

Large B cell lymphoma of domestic animals labels positively with CD79a and CD20 and is negative for CD3. Most large B cell lymphomas can be shown to have surface or cytoplasmic immunoglobulin and most are clonal for lambda light chain. For some subsets of lymphoma, CD20 is a more useful reagent than CD79a, particularly for plasmablastic lymphoma and T cell-rich large B cell lymphoma.

Studies are now underway to specifically define the changes in B cell lymphoma of dogs that are unique to different breeds and subtypes of lymphoma.¹¹ Remarkably, after years of comparing animal lymphomas to those in humans there is now interest in studying large cell lymphoma in the dog in order to gain information of comparative value in treating human lymphoma. This interest arises because it is easier to define a single nucleotide polymorphism (SNP) in relatively inbred purebred dogs than in outbred human species. In large cell lymphoma there are frequent chromosomal alterations involving the 3q27 band that apparently is a translocation involving regions of other chromosomes within the sites of immunoglobulin genes.

The cell of origin is not known in animals but it can be assumed that this is roughly similar to humans where in large B cell lymphoma there is usually hypermutation of the IgH region compatible with origin from a post-germinal center cell. Clonality is now determined by polymerase chain reaction (PCR) application of the VDJ region in dogs and cats, and this is now routinely carried out in a number of institutions.

Clinical Features

Of 50 cases of large cell lymphoma described in dogs, the topography included generalized lymphadenopathy in 18, enteric or abdominal in nine, a single enlarged node in seven, multicentric in four, skin in four, tonsillar in three, spleen in two, mediastinum in two, and central nervous system in one. In 314 cats with large cell lymphoma, there were 85 cases with gastrointestinal involvement, 68 multicentric, 56 mediastinal, 35 in small intestine only, 25 renal, 20 with one or two nodes only, seven in nasal cavity, six with subcutaneous mass, three in skin, three in heart, two in urinary bladder, and one in liver. In 118 cases of equine lymphoma, there were 25 cases of large cell lymphoma with 20 of large centroblastic-type, four of immunoblastic-type, and one diffuse large cleaved-type. Presentation was not known in most of the horses, but one had generalized lymphadenopathy, one had splenic only, and four had enteric involvement. There was little information relating clinical signs to topography in these cases. About one-third of human patients with large B cell lymphoma have symptoms that include recurrent fever and weight loss.

Large B cell lymphoma must be distinguished from lymphomas of similar type and T cell phenotype, with the variation in T cell types bridging most of the cytologic descriptions previously given. Large B cell lymphoma must also be distinguished from indolent lymphomas of intermediate subtypes like MZL. One of the most difficult diagnostic decisions is the distinction between large B cell lymphoma and Burkitt-type lymphoma that is of intermediate size and characterized by a very high mitotic rate and presence of numerous tingibile body macrophages. One of the reasons this distinction is difficult is that there is a large variation in nuclear size in both large B cell lymphoma and in inter-

mediate type Burkitt's lymphoma, so that in morphometric comparisons there is overlap in nuclear volume but not in mean volume or cell size.

Large B cell lymphomas generally progress with no change in cell morphology. There appears to be progression of changes at the molecular level that may relate to the progression and aggressiveness of tumor with time. Unlike benign B cells, where somatic hypermutation permits antibody diversity, in large B cell lymphoma, dysfunction of this process drives disease progression. Changes in the immunoglobulin genes appear to affect both non-translated as well as coding regions leading to changes in amino acid sequence and ultimately malignant transformation.

Treated animals need to be restaged for subsequent management planning. In human cases, doxorubicin-based therapies are frequently used followed by radiation therapy. Even late-stage disseminated large B cell lymphoma in human patients has been followed by apparently long-term remission using combination chemotherapy, with CHOP being the most common initial regimen. Advancing age is considered an important negative factor in treatment of human patients with large B cell lymphoma; gene activation profiling of human lymphomas suggests that patients who have gene expression that mimics reactive germinal centers have a better survival profile than those whose gene profile reflects the pattern of activated blood lymphocytes.

Treatment and survival is currently under study in animals without firm data. One-third of human patients with large B cell lymphoma have night sweats and irregular fever with weight loss at the time of diagnosis. An international prognostic index developed for large B cell lymphoma, divides human cases into stage 0–1 (35%), stage 2–3 (45%) and stage 4–5 (20%). The 5-year survival for stage 0–1 was 73% and for stage 4–5 was 26%. An almost equivalent estimate of survival was determined by measuring only serum lactate dehydrogenase and β -2 microglobulin levels. The rapidity of achieving complete response on first cycles of treatment is prognostically favorable in humans and apparently has equal value in animals. In humans, a high proliferative rate of the tumor cells tends to correlate with poor survival, while retention of a normally functioning immune system is correlated with longer survival. In humans, over expression of the bcl-2 protein is associated with more frequent relapse but not the presence of BCL-2 gene rearrangement.

Burkitt's Lymphoma

Burkitt's lymphoma is a high grade and multicentric B cell lymphoma characterized by diffuse architecture, heavy tingibile body infiltration (starry sky appearance) at low magnification.^{8,16,22,25} Cytologically, cells are of intermediate size with vesicular nuclei, multiple nucleoli, moderate parachromatin clearing, and high mitotic rates. Burkitt's lymphoma was the first human tumor shown to be curable by chemotherapy and the first shown to be associated with the Epstein-Barr virus

(EBV). Burkitt's lymphoma was also the first solid tumor to be associated with a non-random chromosomal translocation and also the first in which genetic deregulation was shown to be associated with an oncogene (*c-myc*). It was also recognized that the geographic distribution of Burkitt's lymphoma in Africa was associated with both the presence of endemic malaria and the mosquito vector. Later the association of chronic immune stimulation related to malaria infection was shown to increase susceptibility to chromosomal injury by EBV.

There are two forms of the Burkitt's lymphoma. The classic type has very uniform cells, described as being of monotonous type (i.e. like "peas in a pod"): this form is very rare in animals. In contrast, the variant type (i.e. Burkitt-like lymphoma), also known as small non-cleaved cell lymphoma, is very common in animals, particularly in dogs.

Pathologic Features

Because the cells of Burkitt's lymphoma are of intermediate type, the only abnormality that is likely to be noticed in the blood is lymphocytes of intermediate size that have nucleoli, and these may be interpreted as reactive.

The typical architecture of lymph nodes in Burkitt-like lymphoma in dogs is of a multifocal proliferation that compresses normal cells between them.³⁶ Benign areas are identified by more darkly stained cells, usually of smaller type, and with a higher number of RBCs present. The foci of proliferation are irregularly coalescing and are characterized by many tingible body macrophages in areas of apparent homogeneous cell proliferation. Cytologically, the cells are of intermediate type with round to oval nuclei 1.5 RBCs in diameter. There are irregular areas of chromatin distribution with parachromatin clearing and 1–4 small but prominent nucleoli. Cytoplasm is moderate in volume and staining density, with cell boundaries generally indistinct. There are usually more than 10 mitoses per 400× field. The numbers of apoptotic cells will almost always equal or exceed the number of mitoses with the number of tingible body macrophages usually 2–3 times the number of mitoses (Fig. 69.20). Nuclei can be oval but are not indented. This relates to this same population of cells being identified in the older working formulation as "small non-cleaved cells." If excisional or incisional biopsy tissue is examined, there are fading germinal centers, usually only with a mantle cell cuff remaining in the subcapsular areas. The cortex appears diffuse with compression of medulla, and medullary sclerosis is not present.

In the classical form of Burkitt's lymphoma, the cells are of monotonously uniform-type lacking the mild anisokaryosis of the Burkitt-like lymphoma. The interface of the cells in fixed and processed tissues usually results in separation of cytoplasmic membranes along hexagonal lines, which is known as the "squaring-off" phenomenon typically seen in human Burkitt-type lymphomas. Architectural characteristics are similar,

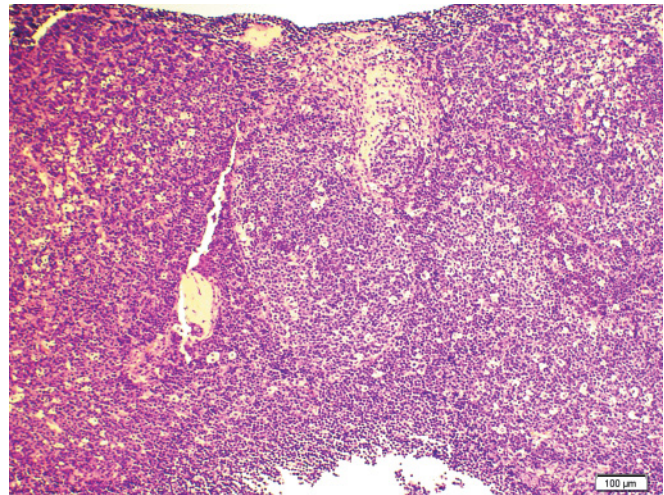


FIGURE 69.20 Lymph node with Burkitt's-like lymphoma. Note the "starry sky" appearance. Wright-Giemsa stain.

although the Burkitt-type usually presents with solid tumor involvement with high tingible body macrophage infiltration, whereas the Burkitt-like lymphoma is more typical of the multifocal areas of proliferation with foci of coalescing tumor cells. The mitotic rate is similar in both tumor types.

Other organs likely to be involved in the dog include the liver, mesenteric lymph nodes, and intestinal wall. Although the spleen may be involved, the histopathology has not been extensively described.

Burkitt-type and Burkitt-like lymphomas stain strongly with CD79a and CD20, and are negative with CD3.^{1,15,26} Currently, specific information on molecular or chromosomal changes in Burkitt-type lymphoma in animals is lacking. Burkitt-type lymphoma in humans has the *c-myc* translocation, whereas the Burkitt-like variant in humans has variable staining for CD10 and Bcl-6 that is strongly positive in Burkitt-type lymphoma and stains positive for Bcl-2 which is not expressed in the classical form.

Burkitt-type lymphoma and Burkitt-like lymphoma must be distinguished from acute lymphoblastic leukemia and lymphoblastic lymphoma.^{34,35} This is largely based on the presence of aggregated chromatin with parachromatin clearing and prominent small nucleoli in Burkitt's tumors rather than the uniformly dispersed chromatin and obscured nucleoli of acute lymphoblastic leukemia and lymphoblastic lymphoma. The Burkitt-type lymphoma is distinguished from Burkitt-like lymphoma on the basis of very uniform cell type in Burkitt-type lymphoma with mild anisokaryosis in the Burkitt-like subtype.

Clinical Features

About 20% of human Burkitt's lymphoma cases have bone marrow involvement and oncologists are now finding that dogs also have early involvement of bone marrow. The general presentation of dogs with Burkitt's

lymphoma is of an animal in good condition that suddenly has reduced appetite and is noticed to have one or more enlarged peripheral lymph nodes. Unlike diffuse large B cell lymphoma, Burkitt-like lymphoma tends to present in dogs less than 10 years of age and generally 5–7 years old with no gender preference.

Burkitt-like lymphoma, as well as Burkitt-type lymphoma, tends to be widespread and extranodal at the time of diagnosis. Careful staging is important to determine all sites of involvement. Restaging is essential to determine new or recurrent sites of tumor involvement. That is usually done by x-ray of the chest and ultrasound examination of the abdomen as well as digital palpation. In human cases, serum lactate dehydrogenase and IL-2 receptor levels correlate well with tumor burden and progressively rise in concert with increasing tumor cell volume. Bone marrow aspirate and core biopsy are indicated if the animals have a nonregenerative anemia and the anemia is not likely to be due to prior treatment.

Treatment of Burkitt-like lymphoma appears to be one of the areas where veterinary oncology is making progress, with long-term survival exceeding 2 years in some cases. Human cases that present in early stages of the disease can be confidently cured with current treatments, and even cases that present as advanced have only slightly less probability of long-term survival.

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Plasma Cell Tumors

ANTONELLA BORGATTI

Multiple Myeloma

Epidemiology
Etiology
Pathogenesis and Disease Evolution
Clinical Manifestations of Disease
Diagnosis
Treatment
Prognosis

Solitary and Extramedullary Plasmacytic Tumors

Clinical Manifestations of Disease
Diagnosis
Treatment
Prognosis
Waldenström's Macroglobulinemia

Acronyms and Abbreviations

BM, bone marrow; BMSC, bone marrow stromal cell; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (Lomustine); CD, cluster of differentiation; CEMP, cutaneous extramedullary plasmacytoma; CT, computed tomography; EMP, extramedullary plasmacytoma; FELV, feline leukemia virus; FIV, feline immunodeficiency virus; HVS, hyperviscosity syndrome; IFE, immunofixation electrophoresis; IFN, interferon; Ig, immunoglobulin; IL, interleukin; IRF, interferon receptor factor; IRF4, interferon receptor factor 4; MGUS, monoclonal gammopathy of unknown significance; MM, multiple myeloma; MRD, myeloma related disorders; MRI, magnetic resonance imaging; NCEMP, non-cutaneous extramedullary plasmacytoma; NFκB, nuclear factor kappa B; OAF, osteoclast activating factor; PBSC, peripheral blood derived stem cell; PET, positron emission tomography; PT, prothrombin; PTT, partial thromboplastin; RANK, receptor activator nuclear factor Kappa B; RANKL, receptor activator nuclear factor kappa B ligand; RBC, red blood cell; SOP, solitary osseous plasmacytoma; SPB, solitary plasmacytoma of bone; SSA, sulfosalicylic acid; TNF-α, tumor necrosis factor-α; VAD, vincristine adriamycin (doxorubicin) prednisone; VBAP, vincristine BCNU adriamycin (doxorubicin) prednisone; VBMCP, vincristine BCNU melphalan cyclophosphamide prednisone; VEGF, vascular endothelial growth factor; VEGF-R1, vascular endothelial growth factor receptor 1; WM, Waldenström's macroglobulinemia.

Plasma cell tumors represent a spectrum of diseases characterized by clonal (predominantly monoclonal) proliferation of immunoglobulin (Ig)-producing plasma cells or B cells.²¹ They encompass clinically indolent conditions such as monoclonal gammopathy of unknown significance (MGUS) and Waldenström's macroglobulinemia (WM) as well as malignant processes, including multiple myeloma (MM) and solitary plasmacytoma (solitary osseous plasmacytoma and extramedullary plasmacytoma). All these disorders share common cytomorphologic features, Ig production patterns, and immune dysfunction.

Five major classes of Ig: IgG, IgA, IgM, IgD, and IgE, are synthesized by normal B cells and plasma cells, whereas atypical plasma cells secrete one of these molecules or, occasionally, only light chain molecules.

When the production of heavy and light chain molecules is unbalanced, the excess of light chain proteins is excreted in the urine (i.e. Bence Jones proteinuria; see Chapter 145). In non-secretory-type myeloma, plasma cells do not secrete these proteins.

MULTIPLE MYELOMA

Epidemiology

Multiple myeloma (MM) is estimated to account for <1% of all malignant tumors occurring in domestic animals and approximately 8% of all hematopoietic malignancies in dogs.^{20,29} The true incidence of MM in cats is unknown, but it appears to be less common in

this species, and it is extremely rare in other domestic animals.^{6,36} In humans, more than 50% of MM patients are older than 70 years. Likewise, older dogs (average 8–9 years)^{29,36} and cats (average 12–14 years)^{14,36} are most commonly affected.

Etiology

Factors associated with the development of MM in companion animals have not been clearly identified. In humans, exposure to ionizing radiation is consistently linked to MM development. Genetic and hereditary factors may play a role in myeloma development. Recurrent infections or antigenic stimulation have been proposed as predisposing factors, although epidemiologic studies have not confirmed this association.

A link between MM and feline leukemia virus (FeLV) or feline immunodeficiency virus (FIV) infections has not been identified, but the diagnosis of disease among siblings suggests a familial association.¹⁴ The role of oncogenes, tumor-suppressor genes, cytokines, and their interaction with the bone marrow microenvironment in the etio-pathogenesis of the disease are currently being investigated in animal models. Overexpression of cell cycle regulators such as cyclin D1 and dysregulation of receptor tyrosine kinase have been implicated in the pathogenesis of plasma cell tumors and MM.² Progression of B cell lymphoma to MM in one dog and of solitary plasma cell tumors to MM in both dogs and cats have been reported.³⁸ MGUS, described as a premalignant condition, rarely progresses to MM.

Pathogenesis and Disease Evolution

Multiple myeloma is characterized by the proliferation of malignant plasma cells or their precursors, arising as a clone from a single cell. It is a germinal center-derived tumor with a B cell phenotype and Ig gene hypermutation and rearrangement. MM is associated with a dysregulation of genes responsible for controlling cell growth and survival, resulting in a multistep process that involves alterations in oncogenes and tumor suppressor genes.

Like other lymphocytes, B cells do not have multilineage potential, but they have the ability to self-renew at multiple steps of development. These properties may be co-opted by malignant plasma cells to maintain a “stem cell” phenotype. Multiple myeloma stem cells also may possess or acquire drug resistance: (1) by maintaining quiescence; (2) by increasing intracellular detoxifying enzymes; or (3) by expression of drug transporters on the surface membrane, as has been demonstrated for brain tumors and leukemic stem cells. Stem cell signaling pathways also may support cancer stem cells; for example, aberrant activation of the Hedgehog signaling pathway has been associated with the development of MM, and its inhibition induces plasma cell proliferation.

It has been hypothesized that a subset of MM may derive from MGUS. Furthermore, monosomy 13 seems

to be implicated in the transition of MGUS to MM.¹ Interactions between cancer cells and microenvironment (bone marrow stromal cells and extracellular matrix) are mediated by myeloma cell-derived adhesion molecules, which are essential for tumor cell localization, growth, and survival. Moreover, increased microvessel density has been demonstrated in human patients with MM compared to patients with MGUS and immunohistochemical studies also have shown vascular endothelial growth factor (VEGF) expression by myeloma cells, thus implying the potential role and clinical relevance of angiogenesis in MM.

While VEGF does not appear to have considerable proliferative effects on myeloma cells, it plays an important role in cancer cell migration and angiogenesis. Indeed, myeloma cells have been shown to express the vascular endothelial growth factor receptor 1 (VEGFR1), which may provide an interesting therapeutic target for emerging anti-angiogenic strategies.

The secretion of tumor necrosis factor- α (TNF- α) by myeloma cells not only stimulates the production by bone marrow stromal cells (BMSCs) of interleukin-6 (IL-6), an essential growth and survival factor for these cancer cells, but also induces nuclear factor kappa B (NF κ B) activation leading to the up-regulation of adhesion molecules.

Clinical Manifestations of Disease

The infiltration of various organ systems by neoplastic cells, the production of cytokines by the tumor or the bone marrow microenvironment, and the high circulating level of a single type of immunoglobulin (M-component), lead to a wide array of clinical manifestations. Multiple myeloma patients are usually immunocompromised and thus highly susceptible to infections. This occurs due to suppression of normal Ig and albumin secondary to a variety of factors, including a direct effect of the monoclonal Ig, bone marrow crowding, decreased T helper cell function, and macrophage-related factors influencing the progression of B cells to plasma cells.

A variety of pathologic conditions are associated with MM, including bleeding diathesis, hyperviscosity syndrome (HVS), renal disease, cardiac disease, skeletal lesions, hypercalcemia, bone marrow hypoplasia, and hypercholesterolemia. The pathogenesis of bleeding diathesis is likely multifactorial. The M-component may interfere with normal coagulation and lead to hemostatic defects by various mechanisms that include: (1) inhibition of platelet aggregation and release of platelet factor 3; (2) adsorption of minor clotting factors; (3) induction of abnormal fibrin polymerization; and (4) functional decrease in calcium. Clinically, hemorrhages occur in approximately one-third of dogs and one-fourth of cats affected by MM.^{14,29,36} In instances where myelophthisis is present due to profound bone marrow infiltration, thrombocytopenia may develop and contribute to hemorrhagic events. Approximately 50% of dogs affected by MM have abnormal prothrombin (PT) and partial thromboplastin (PTT) times. In a recent ret-

respective study of MM in 16 cats, thrombocytopenia was identified in just over 50% of the cats and one had evidence of a prolonged PT.³⁶

Hyperviscosity syndrome is characterized by clinicopathologic abnormalities that occur secondarily to increased serum viscosity, which is associated with the M-component. Hyperviscosity syndrome is most commonly associated with IgM macroglobulinemia due to the high molecular weight of IgM.¹⁰ However, it also can occur in presence of IgA and rarely with IgG.²⁹ Hyperviscosity syndrome leads to bleeding diathesis, neurologic signs (such as seizures, depression, coma), congestive heart failure, renal failure, and ophthalmic abnormalities, including tortuous and dilated retinal vessels, retinal hemorrhages, and retinal detachment, sludging of blood within small vessels, and impaired delivery of nutrients and oxygen to tissues. Approximately 20% of dogs with MM develop HVS and it has been reported in cats.³⁶

Renal disease occurs in approximately 30–50% of dogs and about 30% of cats with MM.^{29,36} The pathogenesis of renal disease is commonly multifactorial and implicated mechanisms include: (1) tumor infiltration within the renal parenchyma; (2) hypercalcemia; (3) amyloidosis; (4) decreased renal perfusion due to HVS; (5) dehydration; (6) ascending urinary tract infections; (7) Bence Jones proteinuria; and (8) development of light-chain casts causing interstitial nephritis.²⁹ Light chain proteins have a low relative molecular mass and are normally filtered through the renal glomerulus. In MM, however, an imbalance between heavy and light chain proteins occurs, which leads to an excess of light chains that can precipitate and cause renal tubular obstruction and injury. In the absence of a concurrent monoclonal spike, light chain proteinuria is most likely indicative of light chain disease. Bence Jones proteinuria has been estimated to occur in approximately 25–40% of dogs and approximately 65% of cats with MM.^{29,36} Urine protein electrophoresis remains the preferred diagnostic modality to detect monoclonal light chain proteinuria.

Heart failure may occur as a result of hyperviscosity-related myocardial hypoxia and increased cardiac workload. In addition, amyloid deposition in the myocardium and anemia may exacerbate the severity of this condition. Approximately 50% of cats with MM in one study had an idiopathic heart murmur.³⁶

Skeletal lesions can be either solitary (well-circumscribed, “punched-out” area of osteolysis) or multiple (diffuse osteopenias) and occur in approximately 50–56% of dogs with MM.²⁹ Rarely, pathologic fractures are seen. Skeletal lesions are typically identified in bones involved in active hematopoiesis (e.g. ribs, vertebrae, pelvis, and proximal and distal aspects of long bones).

Hypercalcemia has been reported in approximately 15–20% of dogs and 20–25% of cats affected by MM.^{14,36,40} It is thought to result from the release of an osteoclast-activating factor (OAF) by either the bone marrow microenvironment or by cancer cells locally in bone.²⁹ Parathyroid hormone-related peptide also may contribute to the pathogenesis of MM-related hypercalcemia.³⁹

In humans, interleukin-1 (IL-1), IL-6, tumor necrosis factor- α (TNF- α) and the receptor activator of the nuclear factor kappa B ligand (RANKL) all modulate osteoclast activity and may contribute to hypercalcemia. Because binding of calcium by the M-component increases the total calcium concentration, determination of ionized calcium is necessary to confirm a true hypercalcemia.

Approximately 30% of dogs and 75% of cats have a normocytic normochromic nonregenerative anemia associated with anemia of inflammatory disease, myelophthisis, hemostatic abnormalities resulting in blood loss, or increased red blood cell (RBC) destruction induced by HVS.²⁹ In humans with MM, decreased erythropoietin responsiveness has been documented. Multiple myeloma-related thrombocytopenia and neutropenia can also be observed in cats and dogs.^{14,36}

Hypocholesterolemia was noted in approximately 69% of affected cats in one study.³⁶ This finding was inversely correlated with globulin concentration and was suspected to be related to down-regulation of hepatic cholesterol production in the attempt to maintain adequate oncotic pressure despite the presence of hyperglobulinemia.

Diagnosis

Symptoms suggestive of MM include the onset of bone pain or back pain, renal failure, anemia, and a history of recurrent infections. Patients exhibiting these signs should be evaluated with a complete blood count, serum biochemical profile, and urinalysis. Serum and urine protein electrophoresis should be performed in the presence of hyperglobulinemia and proteinuria, respectively, to confirm the presence of a monoclonal gammopathy and identify the class of Ig involved in the disease process (see Chapter 145). In patients experiencing bleeding, a hemostatic profile should also be performed and serum viscosity measurements considered (see Chapters 138 and 145). In dogs affected by MM, the incidence of IgA and IgG is comparable, whereas in cats, IgG is most commonly involved.^{29,39,40} Rarely, occurrences of biclonal gammopathies have been reported and, on occasions, cryoglobulinemia has been described in dogs.⁶

Screening for Bence Jones proteinuria should not be based solely upon urine dipstick because this modality primarily detects albuminuria (see Chapter 145). The sulfosalicylic acid (SSA) test provides greater sensitivity for globulin detection but specificity is low due to the concomitant detection of albumin, globulin, Bence Jones proteins, proteases, and polypeptides. In people, false positive results for Bence Jones proteins detected by means of heat precipitation can occur due to excessive amount of polyclonal light chain proteins in patients affected by a variety of conditions, including non-plasmacytic tumors and chronic renal failure. Therefore, urine protein electrophoresis remains the preferred diagnostic modality for the detection of Bence Jones proteins.

Bone marrow infiltration by MM can be quite variable, with the vast majority of patients having an amount of plasma cells greater than 5%. The appearance of malignant plasma cells can range from normal plasma cells, to mildly differentiated cells, to bizarre atypical forms. Plasma cell morphology can be an important predictor of severity of disease (Fig. 70.1).⁸ In humans, histopathologic grade is correlated with disease progression, with grade I disease being slow-growing in contrast to grade III disease (plasmablastic-type) characterized by an aggressive behavior.

Survey radiographs are typically recommended to screen for skeletal lesions. These vary from areas of osteopenia observed in early stages of diseases to lytic, “punched-out” lesions typical of later stages. Biopsy and histopathology of a lytic lesion may sometimes be necessary for a definitive diagnosis. Skeletal lesions are rare in patients with Waldenström macroglobulinemia

where cancer cells more commonly infiltrate other organs, including liver, spleen, and lymph nodes.^{10,16}

In cats, the frequent identification of abdominal organ neoplasia (85% of organs with an imaging abnormality) underscores the need to incorporate abdominal imaging as part of the routine staging (Fig. 70.2).³² A fundoscopic examination should be performed to rule out ocular lesions, such as retinal hemorrhages, retinal detachment, venous tortuosity, dilation, sacculization, and blindness.

Other conditions associated with monoclonal gammopathies that should be considered as differential diagnoses include lymphoproliferative disorders, chronic infections (e.g. ehrlichiosis and leishmaniasis in dogs, and feline infectious peritonitis in cats), immune-mediated disorders, and other neoplastic conditions.

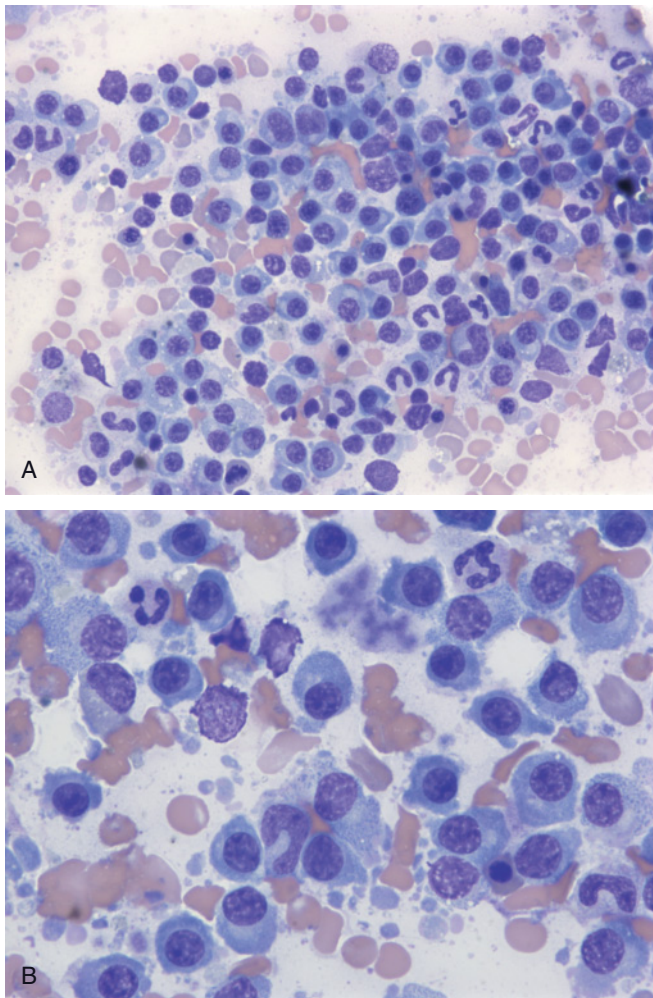


FIGURE 70.1 (A) Canine bone marrow aspirate (500×): Notice the numerous well-differentiated plasma cells admixed with late stage granulocyte precursors and metarubricytes. (B) Canine bone marrow aspirate (1000×): Notice the well-differentiated plasma cells. (Courtesy of Dr. Laura Snyder, University of Minnesota.)

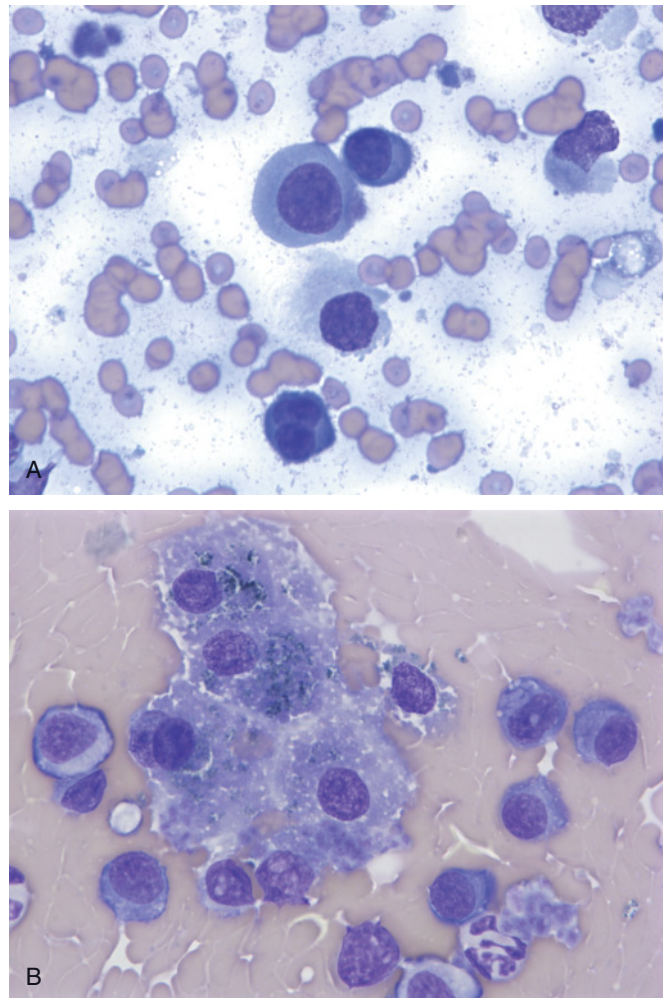


FIGURE 70.2 (A) Feline splenic aspirate (1000×): Notice the well-differentiated plasma cell (top center right), large poorly-differentiated plasma cell (top center left), plasma cell with bilobed nucleus (bottom). (B) Feline hepatic aspirate (1000×): Notice the hepatocytes surrounded by RBCs and plasma cells. (Courtesy of Dr. Laura Snyder, University of Minnesota.)

Treatment

Treatment of MM with oral melphalan and prednisone is the standard of therapy due to its dual ability to reduce the bulk of the tumor and the symptoms of disease. Although complete eradication of disease is only rarely achieved, chemotherapy is often effective in decreasing tumor burden, reducing serum Ig levels, promoting bone remodeling, and providing symptomatic relief. Overall this leads to improved quality and possibly duration of life.^{29,30}

Melphalan is an alkylating agent whose oral absorption is unpredictable, requiring administration to be made preferably on an empty stomach. In dogs, melphalan is initially administered at 0.1 mg/kg orally once a day for 10 days, and then at 0.05 mg/kg orally every other day. Prednisone is generally administered at 1.0 mg/kg for the first 10 days of therapy, and then decreased to 0.5 mg/kg every other day. Treatment is continued indefinitely, until relapse or myelosuppression occurs. Possible myelosuppression is generally monitored via serial complete blood counts performed biweekly for 2 months and monthly thereafter. A pulse-dosing regimen, where melphalan is administered at 7 mg/m² orally for five consecutive days every 3 weeks, has been used successfully in patients experiencing dose-limiting thrombocytopenia with the more conventional low-dose treatment described above.

Other alkylating agents including cyclophosphamide (Cytosan), chlorambucil (Leukeran), and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU or Lomustine) also have been used to treat MM in dogs and cats.⁷ The addition of cyclophosphamide to a prednisone/melphalan regimen may be beneficial to patients with severe clinical signs and/or hypercalcemia. Due to its platelet-sparing effect, cyclophosphamide may be used in place of melphalan in thrombocytopenic patients, although this drug can have severe suppressive effects on other bone marrow lineages. Chlorambucil, administered at 0.2 mg/kg orally once daily has been used successfully for the treatment of IgM macroglobulinemia in dogs. CCNU has been used in cats at 50 mg/m² orally every 21 days, resulting in one partial response.⁴¹ Combination chemotherapy protocols incorporating vincristine, carmustine (BCNU), melphalan, cyclophosphamide, and prednisone (VBMCP) or vincristine, melphalan, cyclophosphamide, and prednisone/vincristine, BCNU, doxorubicin, and prednisone (VMCP/VBAP) have been extensively utilized in people, but outcomes are essentially comparable to those of patients treated with prednisone and melphalan alone.

Glucocorticoids have been shown to induce apoptosis *in vitro* via inhibition of I κ B activation and decreased NF κ B activity. The administration of high-dose dexamethasone in conjunction with vincristine and doxorubicin (VAD) was investigated in humans with refractory myeloma and resulted in a response rate greater than 50%. Rapid tumor response, alleviation of bone pain, resolution of hypercalcemia, and absence of cumulative

damage to bone marrow stem cells were remarkable advantages of this treatment combination.

Anecdotally, in dogs, responses of a few months duration have been achieved with a combination of doxorubicin (30 mg/m² intravenously every 21 days), vincristine (0.7 mg/m² intravenously on days 8 and 15), and prednisone (1 mg/kg orally daily). Rescue approaches have been taken in dogs with refractory myeloma with high-dose cyclophosphamide (limited success), and liposome-encapsulated doxorubicin, which resulted in a durable remission in one dog.¹⁸

The efficacy of interferon (IFN) for the treatment of MM is controversial. While a response rate of approximately 20% was reported in humans with relapsed myeloma, the addition of IFN to standard chemotherapy approaches failed to provide a significant benefit to the overall survival time in a meta-analysis of 2,286 patients.²⁷

High-dose chemotherapy in association with autologous transplantation using bone marrow (BM) or blood-derived stem cells (PBSCs) is now widely used for the treatment of hematological malignancies including MM (see Chapter 102). This approach yielded complete remissions in refractory human patients, but mortality rate due to bone marrow suppression was high. Contamination of most bone marrow and blood stem cell samples with neoplastic cells has been shown in MM even after high-dose chemotherapy. The presence of tumor cells within the autograft has resulted in recurrence of disease, emphasizing the need to optimize purging techniques.

In humans with indolent myeloma, treatment is generally withheld until disease progression, symptomatic manifestations, or new lytic bone lesions develop. However, bisphosphonates such as pamidronate have been used to prevent or delay the onset of bone lesions and associated bone pain.

Treatment of patients affected by indolent myeloma with the anti-angiogenic agent thalidomide resulted in a 66% response rate and the drug appeared to have potential to delay the onset of clinical signs associated with the disease. The efficacy of thalidomide for the treatment of refractory relapsed MM has also been confirmed. The combination of thalidomide and dexamethasone has yielded response rates greater than 50% in patients with relapsed myeloma and 70% in newly diagnosed patients.³⁵ Studies evaluating the role and possible efficacy of thalidomide for the treatment of MM in pet animals are lacking, in part because federal regulations in the United States prohibit dispensing thalidomide for veterinary patients.

Bortezomib, a proteasome inhibitor, induces apoptosis of MM cells and inhibits their binding to BMSCs, which otherwise would trigger the transcription of IL-6 via an NF κ B-dependent pathway.¹⁵ In a multicenter phase II trial of bortezomib, a 35% response rate was achieved with a median duration of remission of 12 months and an overall survival time of 16 months. Addition of dexamethasone to the treatment regimen improved responses in 19% of treated patients.

Response to therapy is assessed based on amelioration of clinical signs as well as improvement in the radiographic appearance of skeletal lesions and clinicopathologic abnormalities.²⁹ Because complete resolution of MM is extremely rare, a $\geq 50\%$ reduction in the pre-treatment M-component (Bence Jones proteins or Ig) is considered a good response to therapy.

Patients experiencing severe clinical signs secondary to hypercalcemia and renal impairment, HVS, or pathologic fractures may require additional therapy specifically directed to the clinical complications of disease. For example, fluid therapy and diuretics may need to be instituted for patients with severe hypercalcemia. Similarly, plasmapheresis may be beneficial in minimizing clinical signs of hyperviscosity, while aggressive fluid therapy may be necessary for patients with renal failure.

Prognosis

In humans with MM, the course of disease is extremely variable, with survival times ranging from less than 1 year with aggressive forms to more than a decade in indolent forms. Prognostic variables in people include: (1) tumor burden (serum calcium, immunoglobulin, albumin, α_2 -microglobulin, number of lytic bone lesions, percentage of bone marrow plasmacytosis); (2) tumor biology (mitotic rate, IgA myeloma, plasma cell labeling index); (3) tumor microenvironment (bone marrow microvessel density and matrix metalloproteinase 9 level, among others); (4) treatment (12 months or less of prior treatment, achievement of complete remission, tandem transplantation, second transplantation within 6 months); and (5) patient (performance status, albumin level, other unrelated conditions).

α_2 -Microglobulin has been consistently identified as a predictor of survival in MM. It is a light-chain protein belonging to the class I of histocompatibility antigens expressed on the surface of nucleated cells and released into the blood. Because α_2 -microglobulin is excreted by the kidneys its level reflects tumor burden as well as renal function.

In humans, a nodular pattern of bone marrow infiltration appears to be associated with a worse prognosis compared to a diffuse pattern. In addition, severity of disease has been correlated with cell morphology. For example, plasmablastic disease has been associated with a more aggressive behavior of the cancer.

In dogs with MM, the short-term prognosis is good in respect to initial tumor control and improvement of quality of life. However, the long-term prognosis is generally poor due to high likelihood of tumor relapse and recurrence of clinical signs as well as development of resistance to chemotherapeutic agents. In a study of 60 dogs with MM treated with melphalan/prednisone combination chemotherapy: 8% did not respond to therapy; 43% achieved complete remission (defined as normalization of serum Ig); and 49% achieved a partial remission (defined as less than 50% decrease in the pre-treatment Ig level). The median survival time was 540

days. Negative prognostic factors identified in this study include presence of hypercalcemia, extensive bone lysis, and evidence of Bence Jones proteinuria.²⁹

Short-term prognosis is unfortunately not as good in cats as it is in dogs. Although the majority of cats initially respond to melphalan/prednisone or cyclophosphamide-based chemotherapy regimens, responses are often partial and short-lasting. The median survival of cats is approximately 4 months, but survival times greater than 1 year have been reported.^{5,14,29,40}

IgM macroglobulinemia has been identified and treated only in a small number of dogs. In nine dogs treated with chlorambucil, 77% achieved remission and the reported median survival time was 11 months.

SOLITARY AND EXTRAMEDULLARY PLASMACYTIC TUMORS

Extramedullary plasmacytoma (EMP) is a neoplastic plasma cell tumor originating from soft tissues in the absence of primary bone marrow disease; both cutaneous (CEMP) and non-cutaneous (NCEMP) forms have been described.^{24,27} Solitary osseous plasmacytoma (SOP or SPB) is an isolated lesion of bone origin with potential to progress to systemic MM.^{23,31} Plasma cell tumors account for approximately 2.4% of all canine tumors and most commonly occur at cutaneous sites (86% of cases), followed by mucous membranes of lips and oral cavity (9%), colon, and rectum (8%). Approximately 22–28% of EMPs in dogs are located within the oral cavity.⁴ Feline EMPs have been reported to occur in skin, upper lip, gingiva, orbit, gastrointestinal tract, brain, and retroperitoneal space, and their incidence appears to be low.^{3,12,22,25,26,33}

The median age of dogs affected by plasmacytomas is 9–10 years and breed predispositions include American cocker spaniel, English cocker spaniel, and West Highland White terrier. The median age of affected cats is 10.6 years and the European short-haired cat is overrepresented.^{22,37} Individual cases have been reported in Abyssinian, Persian, and Burmese breeds.

Historically, EMP carries a better prognosis than MM. However, the biological behavior of EMP highly depends upon its anatomic location. For example, EMPs in cutaneous and oral cavity locations are generally associated with a low metastatic rate, and thus are curable by means of surgery alone. Progression of cutaneous EMPs to systemic disease has been documented in two cats, one of which developed MM, the other nodal and distant metastatic disease.^{3,38} Non-cutaneous and non-oral forms of EMPs typically have a more aggressive behavior. Gastrointestinal EMPs, affecting esophagus,¹³ stomach,²⁴ small intestine,¹⁷ and large intestine^{17,19,24} are frequently associated with lymph node metastases, although monoclonal gammopathies and bone marrow involvement are rare.

Solitary osseous plasmacytoma has been reported to occur in ribs and zygomatic arch of dogs. Solitary osseous plasmacytoma frequently progresses to MM, although this may take months to years to occur.^{24,31}

Clinical Manifestations of Disease

Clinical manifestations are dependent on the anatomic location of the tumor. Cutaneous forms generally manifest as a solitary, raised, alopecic, smooth nodule 1–10 cm in diameter, and clinical signs of illness are typically absent. Bleeding from the mouth was the chief complaint in canine oral EMP. Gastrointestinal EMP is associated with nonspecific signs, whereas colorectal EMP often results in tenesmus, rectal prolapse, rectal bleeding, and hematochezia.¹⁹ Pain, lameness, and neurologic signs may be observed in dogs with SOP affecting the appendicular or the axial skeleton.

Diagnosis

Fine needle aspiration or biopsy is needed to confirm a diagnosis of SOP or EMP. Reclassification of reticulum cell sarcomas as plasmacytomas has resulted in an increased incidence of this type of tumor in veterinary medicine.⁴

Plasmacytomas are generally characterized by a monomorphic population of plasma cells with occasional binucleate or multinucleate cell present. A polymorphic blastic plasma cell that appears to be associated with a more aggressive behavior in the dog has also been described.^{2,19} Immunohistochemical detection of cytoplasmic Igs with predominant clonality for κ - or λ -chains is a common finding and 97–100% of EMPs are positive for λ -chain reactivity.²⁴ Moreover, monoclonal light-chain reactivity is helpful in differentiating plasmacytomas from other round cell tumors. However, Ig heavy chains (IgG and IgA chains) have been confirmed in other feline tumor cells; which often contain amyloid deposits.^{3,22,26,37} Canine plasmacytomas stain positively for IgG, A, or M as well as CD45, CD18, CD45RA, and CD79a (Mb-1) leukocyte antigens. While the Cyclin-D1 marker has been identified in MM, no association has been found that relates Cyclin-D1 to plasmacytomas. Interestingly, the identification of this marker in EMPs has been postulated to be a possible indicator of a highly-aggressive malignancy or a metastatic lesion from MM.²

A complete staging should be performed before initiation of therapy to rule out systemic involvement, which, if present, would alter treatment recommendations and prognosis. Diagnostic modalities should include serum protein electrophoresis, skeletal survey radiographs, bone marrow aspiration, and cytology, particularly for SOPs and gastrointestinal EMPs due to their higher metastatic potential.

Treatment

Surgery is considered the treatment of choice for cutaneous plasmacytomas and, on rare occasions, melphalan and prednisone therapy has been successfully utilized in canine and feline cases where surgery resulted in incomplete tumor margins or local tumor recurrence. Surgery is also recommended in patients with SOPs associated with a pathologic fracture or with

neurologic compromise. Spinal cord decompression combined with removal of the primary tumor and spinal cord stabilization may need to be performed in selected patients. The efficacy of radiation therapy in palliating bone pain associated with the tumor itself and/or the presence of pathologic fractures has been reported; however, the majority of patients eventually developed MM.^{23,31}

In patients with SOPs and no evidence of systemic involvement, it is debatable as to whether chemotherapy should be instituted at the same time as local therapy. Likewise, gastrointestinal EMPs are managed solely with surgery, unless systemic disease is documented, in which case the incorporation of chemotherapy would be warranted. Mandibulectomy or maxillectomy is recommended for treatment of oral plasmacytomas accompanied by bony infiltration.⁴ All dogs with oral plasmacytomas excised with incomplete surgical margins experienced local tumor regrowth in one study.⁴¹ Periodic monitoring and restaging are pivotal in allowing early detection of tumor regrowth and metastatic disease.

Prognosis

Oral, cutaneous and mucocutaneous plasmacytomas are associated with a good prognosis and can be cured by means of surgery alone, provided complete surgical margins are achieved.^{2,41} Anecdotally, multiple plasmacytomas that eventually resulted in death of affected dogs have been reported. Tumor cell proliferation rate (based on Ki-67 staining) and histopathologic grade have not been found to hold prognostic significance in dogs or cats, although a more aggressive behavior has been attributed to a polymorphic blastic type of canine plasmacytoma.^{2,25} Cyclin-D1 expression and presence of amyloid also do not seem to have clinical significance.²

Radiation therapy is the preferred treatment option for solitary plasmacytomas. In fact, local recurrence rates less than 10% and long-term survival in 30% and 70% of patients with SOPs and EMPs, respectively, are expected in patients treated with definitive courses of radiation therapy. This is most likely due to the high-grade nature of round cell tumors that makes them susceptible to therapy.

Long-term survival times have also been reported for dogs with gastrointestinal EMPs or EMPs affecting liver, uterus, or other abdominal organs, undergoing surgery and/or chemotherapy.^{19,23,24} The median survival time of nine dogs with colorectal plasmacytomas was 15 months;¹⁹ two dogs treated with surgery alone experienced tumor regrowth 5 and 8 months post-operatively. DNA aneuploidy and c-myc oncogene content of canine plasmacytomas were analyzed by means of flow cytometry and found to be predictive for survival; however, findings strongly correlated with non-cutaneous forms of disease, suggesting that location of the tumor is a prognostic indicator of equivalent importance.⁹

In cats, prognostic information is limited mostly due to the rarity of these tumors and the related paucity of

cases reported in the literature. In general, principles of therapy that apply to dogs should be also applied to feline patients.

WALDENSTRÖM'S MACROGLOBULINEMIA

Waldenström's macroglobulinemia (WM) is a rare lymphoproliferative, immunosecretory disorder occurring in dogs, cats, and humans. According to the WHO classification scheme, it is considered a form of lymphoplasmacytic lymphoma that involves spleen, bone marrow, and lymph nodes and can manifest with IgM-related signs of cryoglobulinemia or hyperviscosity. This description corresponds to the lymphoplasmacytic type of immunocytoma incorporated into the Kiel classification and to the REAL lymphoplasmacytoid lymphoma/immunocytoma. A consensus has not yet been reached among pathologists in regard to the new classification, most likely due to the subjectivity involved in the distinction between small or plasmacytoid lymphocytes and plasma cells.

Waldenström's macroglobulinemia is characterized by bleeding tendencies, anemia, lymphadenopathy, high-serum viscosity with a high molecular mass M-component, and is associated with a monoclonal expansion of B cells infiltrating the bone marrow or other lymphoid organs producing an IgM serum paraprotein. IgM consists of high-relative molecular mass, positively charged proteins that can bind to RBCs leading to rouleaux formation. Waldenström's macroglobulinemia differs from MM in that bone lysis is generally absent and hepatosplenomegaly, lymphadenopathy, or both are frequently encountered clinical findings. Peripheral neuropathy, hyperviscosity syndrome (HVS), and HVS-associated complications that include retinal hemorrhage, epistaxis, cardiac and renal damage, and central nervous system abnormalities can occur. In addition, IgM can cause immune-mediated disorders such as cryoglobulinemia, cold agglutinin hemolytic anemia, and peripheral neuropathies secondarily to autoantibody activity.^{11,34} Bleeding diathesis has been reported to occur in approximately 33% of affected dogs and it is suspected to derive from the interference of IgM with clotting factors and/or platelet aggregation. Possible laboratory findings in both humans and dogs include anemia, pancytopenia, leukocytosis or leukopenia, hypercalcemia, IgM hypergammaglobulinemia, and Bence Jones proteinuria.¹¹ In addition to WM, the production of IgM paraproteins has been reported in association with MM, chronic lymphocytic leukemia, splenic marginal zone lymphoma, extranodal marginal zone lymphoma, plasmacytoid lymphoma, and non-neoplastic conditions such as leishmaniasis.

In human patients, findings that may aid in differentiating WM from other non-neoplastic monoclonal gammopathies or MGUS include the degree of bone marrow infiltration and the amount of M-component, and it has been suggested that the minimum threshold of IgM level is 30 g/L and the percentage of plasma cells

in bone marrow is 20%.²⁰ Diagnostic features of this disease in dogs are still a matter of debate. Immunophenotyping and serum immunofixation electrophoresis (IFE) are often necessary to more definitively diagnose lymphoproliferative disorders in pet animals.

Treatment of WM is unlikely to provide a cure; rather, it is directed at diminishing tumor burden and alleviating clinical signs associated with the disease. Approximately 77% of dogs with IgM macroglobulinemia respond favorably to prednisone therapy combined with alkylating agent-based chemotherapy, and median survival times range from 11 months to 14 months.²⁸ Chlorambucil is most frequently used in conjunction with prednisone, but melphalan and cyclophosphamide have also been shown to have activity against IgM macroglobulinemia.^{11,20}

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Hodgkin's Lymphoma

V.E. TED VALLI

Definition

Classification of Hodgkin's Lymphoma

Pathologic features

Lymphocyte Predominant Type

Lymphocyte-Rich Classic Hodgkin's Lymphoma

Nodular Sclerosis Hodgkin's Lymphoma

Mixed Cellularity Hodgkin's Lymphoma

Lymphocyte Depletion Hodgkin's Lymphoma

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Immunologic Features

Differential Diagnosis

Transformation and Prognosis

Treatment and Survival

Acronyms and Abbreviations

NK, natural killer; RBC, red blood cell; RS, Reed-Sternberg; WHO, World Health Organization.

DEFINITION

Hodgkin's lymphoma is a disease in humans with a number of subtypes all of which require the presence of the iconic Reed-Sternberg cells which themselves vary between the subtypes of this neoplasm. Briefly stated, the World Health Organization (WHO) classification for Hodgkin's lymphoma includes four subtypes: (1) nodular lymphocyte predominance; (2) lymphocyte-rich classic; (3) nodular sclerosis grades I and II; (4) mixed cellularity and lymphocyte depletion.

CLASSIFICATION OF HODGKIN'S LYMPHOMA

Like the non-Hodgkin's lymphoma, the classification of Hodgkin's type of lymphoid neoplasms has been the subject of continuous variation based on new developments and increased understanding of the underlying neoplastic processes (see Chapter 64). Reed-Sternberg (RS) cells are derived from B cells that have a rearranged variable region of the immunoglobulin gene. In type 3 Hodgkin's disease, Reed-Sternberg cells are clonal, whereas in the lymphocyte predominance type, the variable region of the immunoglobulin gene continues to mutate: this is now identified as the lymphocyte-rich classic type. This situation is even more complex with a small proportion of human cases having appar-

ent Reed-Sternberg cells that appear to be dendritic cells or natural killer (NK) cells. Therefore, the definition of Hodgkin's lymphoma in humans is neither entirely a specific or exclusive designation and it becomes more likely that there are types of neoplasms in animals that have characteristics similar to human Hodgkin's lymphoma without being exact replicas of human Hodgkin's lymphoma.¹⁻⁷

T cell-rich large B cell lymphoma in the cat is biologically similar to Hodgkin's disease. Feline T cell-rich large B cell lymphoma characteristically appears first in the nodes in the cervical region, frequently on the right side. Spread from that area will be to a node immediately beneath the initially involved node and secondly either to the prescapular node or down the neck to the mediastinal node.

A further complication is that Reed-Sternberg-type cells in animals with lymphoma differ from those described in humans. Human Reed-Sternberg cells are described as having peripheralized chromatin with a hyperdistended central nucleolus in a binucleated cell arising within "an appropriate cellular background". Most cases of putative Reed-Sternberg cells in animals do not have peripheralized chromatin or hyperdistended nucleoli, even if they are binucleated. Despite that, there are sufficient similarities occurring in the feline type of Hodgkin's-like lymphoma that the designation appears appropriate when considered along with the similarities in biology of the disease.

PATHOLOGIC FEATURES

Lymphocyte Predominant Type

About 5% of human cases of Hodgkin's lymphoma are of the lymphocyte predominant type. Most cases are nodular in type while a small subset is diffuse lymphocytic or diffuse histiocytic in type (Fig. 71.1). The disease is most common in men in the fourth decade of life who present with persistent nodal enlargement in the early stages of the disease, which tends to run a relatively long and indolent course. Most of the cells are small lymphocytes with some histiocytes and in the nodular forms about half of the small lymphocytes are B cells. This subtype of lymphoma is characterized by multinucleated Reed-Sternberg cells known as popcorn-type cells. A consistent feature of this disease is the small lymphocytes ringing the popcorn-type Reed-Sternberg cells referred to as a rosetting pattern. The absence of mononuclear-type Reed-Sternberg cells is considered an important diagnostic criterion.

Lymphocyte-Rich Classic Hodgkin's Lymphoma

The classic-type of lymphocyte-rich Hodgkin's lymphoma now includes all other types that are linked by having the same phenotype of constituent cells, with the differences occurring in topographical areas involved and levels of fibrosis. Lymphocyte-rich classic Hodgkin's lymphoma is defined as a diffuse lymphoid proliferation with infrequent Reed-Sternberg cells (classic-type with very large nucleoli). The accompanying population of small lymphocytes and occasional eosinophils and plasma cells mean the main differential diagnosis is the T cell-rich large B cell lymphoma.

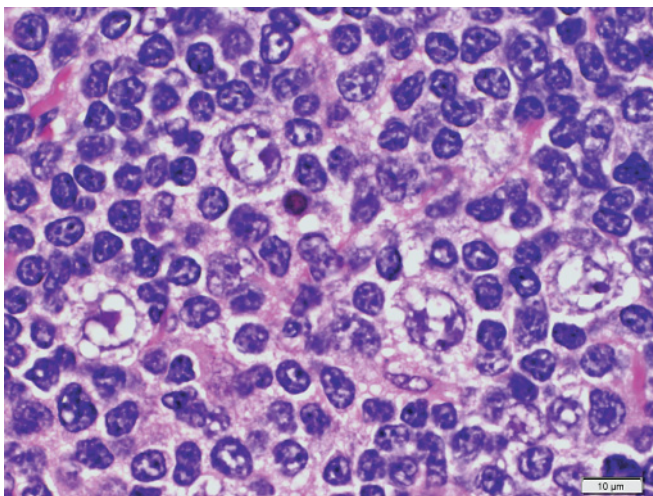


FIGURE 71.1 Human lymph node with Hodgkin's lymphoma: lymphocyte predominant type. The lymphocytes are of small and intermediate type with a predominance of "popcorn-type" Reed-Sternberg cells. The tissues are supported by a fine sclerosis. H&E stain. (Slides from Cornell tutorial.)

Nodular Sclerosis Hodgkin's Lymphoma

This is the most common type of lymphoma in humans, constituting more than 60% of cases, and has the highest level of agreement on review by different diagnosticians. This type of lymphoma is more common in women (generally less than 50 years old) and tends to present in lower cervical, superclavicular, and mediastinal lymph nodes. These incidentally are the comparative age, gender, and sites of presentation of T cell-rich large B cell lymphoma of cats. The dominant factors in diagnosis are broad bands of collagenous connective tissue separating areas of lymphoid proliferation (Fig. 71.2). The background cell population is variable and mixed with small and medium lymphocytes with relatively numerous eosinophils, neutrophils and a fine supporting fibrovascular network. The Reed-Sternberg cells are relatively numerous and are of the lacunar-type, so named because in formalin-fixed tissues the broad cytoplasm of these cells separates from the surrounding tissues leaving the cells in isolated lacunae. These cells frequently have eosinophilic cytoplasm as the cells undergo necrosis. A similar process is seen in cats with T cell-rich large B cell lymphoma.

In the recent WHO classification the nodular sclerosis-type of Hodgkin's lymphoma was divided into two types with type 1 having the majority of cases and type 2 having focal areas of lymphocyte depletion and foci of anaplastic lacunar cells. Patients with type 2 nodular sclerosis Hodgkin's lymphoma have poor response to treatment, increased relapse rate, and shortened survival times.

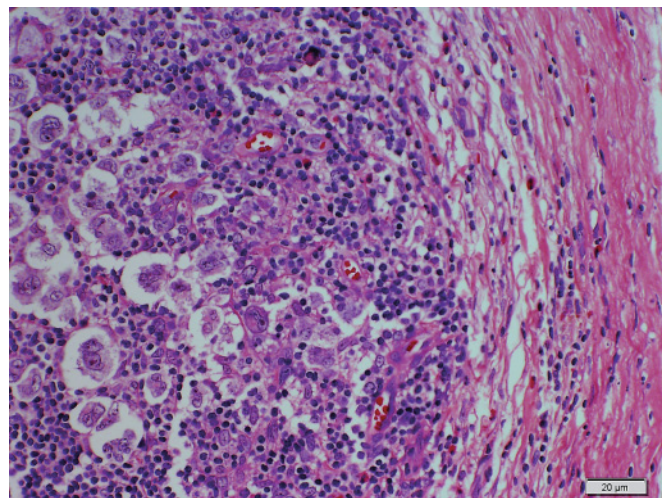


FIGURE 71.2 Human lymph node with Hodgkin's lymphoma: nodular sclerosis type. Foci of lymphoid proliferation are accompanied by large numbers of lacunar-type Reed-Sternberg cells (left center; note areas of peripheral clearing). Note the prominent nucleoli in several of the Reed-Sternberg cells. The right side of the image is made up of broad bands of dense mature collagen separating the lymphoid nodules. H&E stain. (Slides from Cornell tutorial.)

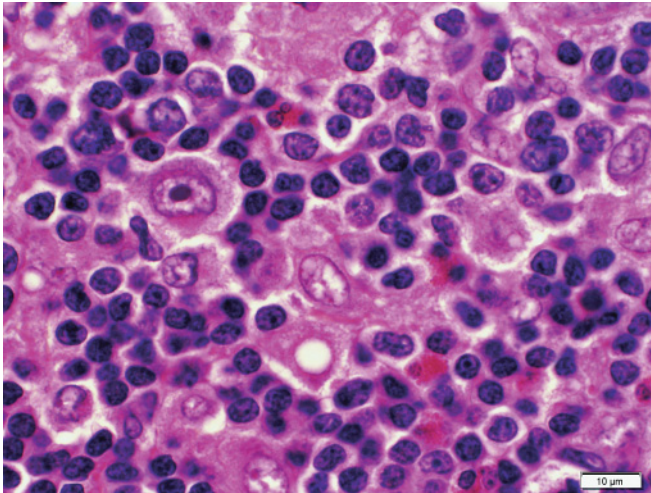


FIGURE 71.3 Human lymph node with Hodgkin's lymphoma: mixed cellularity type. The background population is composed of small lymphocytes with dense nuclei that generally lack nucleoli and appear morphologically and biologically normal. Notice the numerous eosinophils in lower right. The Reed-Sternberg cells are of the classic type with hyperdistended nucleoli and frequent binucleation. H&E stain. (Slides from Cornell tutorial.)

Mixed Cellularity Hodgkin's Lymphoma

This subtype is the second most common type of Hodgkin's lymphoma in humans and is the most common type in males. The condition presents with systemic signs and diagnostically is considered to lie between lymphocyte predominance-type and lymphocyte depletion-type. As the name implies, the background cells are a mixture of small lymphocytes with variable numbers of neutrophils, eosinophils, plasma cells, and macrophages often in clusters (Fig. 71.3). Small lymphocytes have round nuclei with compact chromatin, lack nucleoli, and appear morphologically benign. There is only fine fibrosis but foci of necrosis are frequently present. The differential diagnosis for this type of Hodgkin's lymphoma is broad because many other neoplastic and benign conditions share the general features of mixed cellularity. The Reed-Sternberg cells of the mixed cellularity Hodgkin's lymphoma tend to be of the classic type with binucleation and prominent nucleoli.

Lymphocyte Depletion Hodgkin's Lymphoma

This is the least common type of Hodgkin's lymphoma affecting humans and comprises about 1% of cases. The condition affects older individuals that present with systemic signs and are usually in stage 3 or 4 disease progression. These patients frequently have fever and have suffered marked loss of body mass due to hepatic dysfunction, but generally do not have peripheral lymphadenopathy. The disease tends to be extensive below the diaphragm, involving liver, spleen, retroperitoneal nodes, and bone marrow. This form of Hodgkin's lymphoma

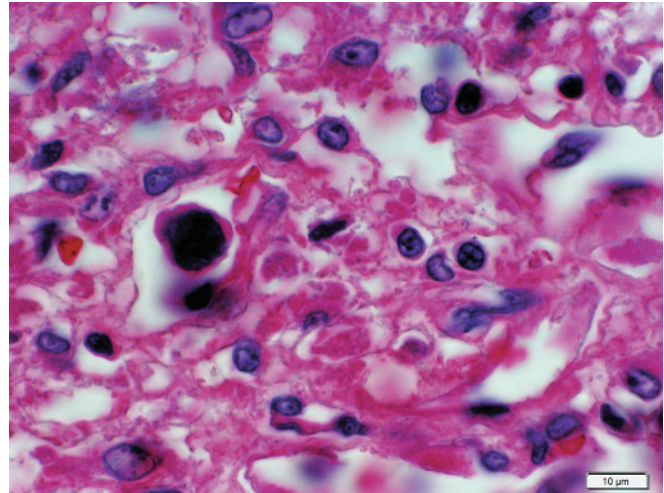


FIGURE 71.4 Human lymph node with Hodgkin's lymphoma: lymphocyte depletion with diffuse fibrosis type. There is marked sclerosis with most of the cells present of connective tissue type and only a few lymphocytes remaining in right center. A moderate number of lacunar-type Reed-Sternberg cells (left center) are atypical with a hyperchromatic nucleus that obscures internal detail. H&E stain. (Slides from Cornell tutorial.)

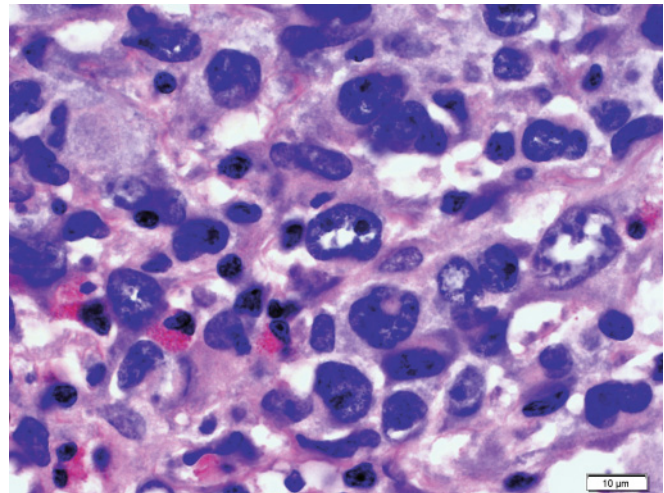


FIGURE 71.5 Human lymph node with Hodgkin's lymphoma: lymphocyte depletion of reticular type. There is a low level of lymphocytes with numerous eosinophils and many atypical Reed-Sternberg cells. The elongated nuclei in the background make up the connective tissue component. H&E stain. (Slides from Cornell tutorial.)

phoma has the shortest survival and survival is poorest in patients that have atypical Reed-Sternberg cells known as reticular type (previously termed Hodgkin's sarcoma; Figs. 71.4 and 71.5). Architecturally, the tissues are characterized by diffuse fibrosis of non-mature collagenous connective tissue with cell depletion mainly involving small lymphocytes. The Reed-Sternberg cells may occur in clusters of the more common type or the anaplastic type.

HODGKIN'S-LIKE LYMPHOMA IN ANIMALS

The most obvious counterpart of Hodgkin's lymphoma in animals is the T cell-rich large B cell lymphoma most common in horses and cats, with that in the cat being best described and understood. In the cat, T cell-rich large B cell lymphoma tends to present with an enlarged node in the cervical area that may be as long as 4 cm, half that in width and 1 cm in depth. A consistent characteristic of these tumors is that they are very cohesive with a fine dense fibrovascular supporting structure and with the nodes remaining mobile beneath the skin except when they become very large. As a consequence these tumors can be effectively removed in their entirety.

The architecture may be uniform throughout the entire nodal profile or there may be focal areas with apparent tumor involvement and other areas with fading germinal centers. The lesions are very slow growing and the animals tend to retain normal appetite and activity. The tumor may recur in exactly the same place 6 months or more after initial removal. This is due to another small node in the same region having become involved.

The cytologic appearance of the nodes is variable but always of mixed cell type with the absence of a homogeneous population of cells that would normally be required for diagnosis of lymphoma. The cells present are small, intermediate, and large lymphocytes that make up the atypical population (Fig. 71.6). Lymphocytes may be as low as 5–10% of the total cells present having round to oval nuclei 2–4 red blood cells (RBCs) in diameter with fine dense, often peripheralized, chromatin and with a prominent single central nucleolus. Like the

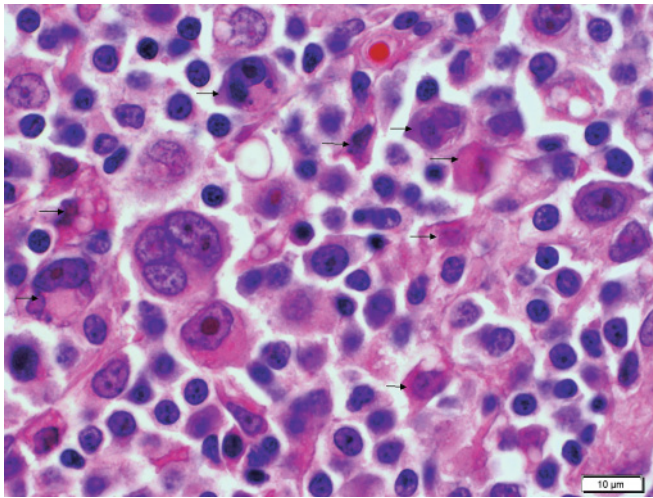


FIGURE 71.6 Cat lymph node with T cell-rich large B cell lymphoma. The predominant population is small lymphocytes with some intermediate-sized lymphocytes. The atypical cells have large nuclei with prominent nucleoli and are occasionally multinucleated. Many of the atypical cells (arrows) have eosinophilic cytoplasm and are undergoing single cell necrosis typical of Reed-Sternberg cells in this type of lymphoma. H&E stain.

human lacunar cells, feline large neoplastic cells frequently contract from the surrounding tissues which also makes them visible on low power examination. In addition, the cytoplasm is frequently deeply basophilic due to the cells undergoing necrosis.

Immunologic Features

In the cat, only 5–10% of the large atypical cells stain with CD79a (i.e. B cell marker) but most of the cells stain with CD20 (i.e. B cell marker; Figs. 71.7 and 71.8). Background cells stain with CD3 (pan T cell marker)

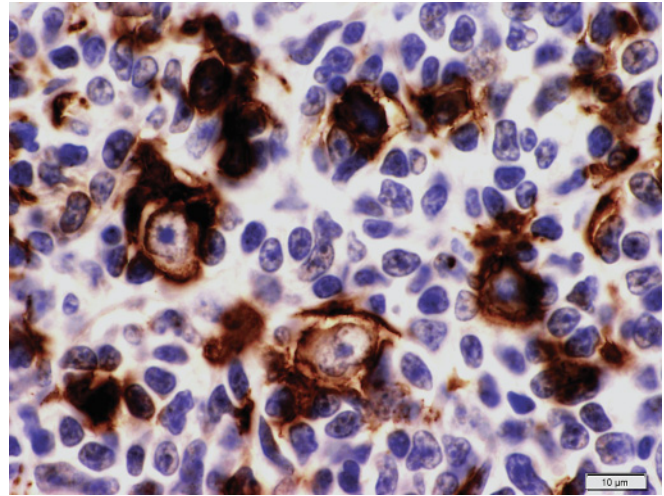


FIGURE 71.7 Cat lymph node with T cell-rich large B cell lymphoma. The atypical cells are strongly positive with the background of small and intermediate size lymphocytes unlabeled. CD20 immunostaining.

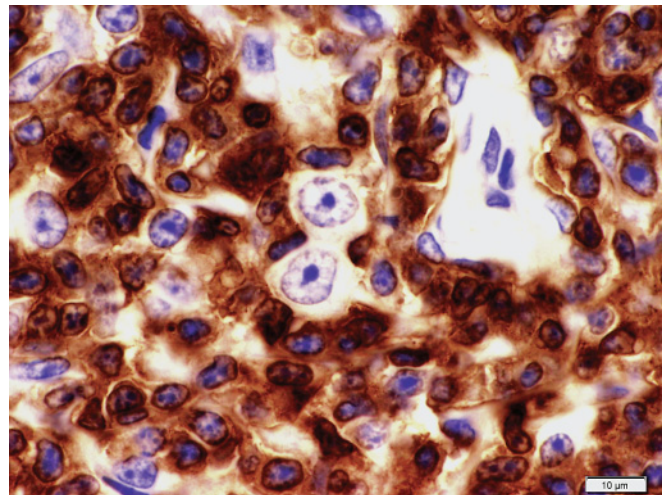


FIGURE 71.8 Cat lymph node with T cell-rich large B cell lymphoma (same case as Figure 71.7). The atypical cells (center) are unlabeled with the background T cells being strongly and uniformly labeled. CD3 immunostaining.

and on gross inspection of immunophenotypic stains it may appear that the tissues are positive for both B and T cell markers. The dense but fine background connective tissue population is unobtrusive but becomes very apparent in nodes stained with reticulin stains.

Differential Diagnosis

A major differential diagnosis in cats with T cell-rich large B cell lymphoma is benign hyperplasia. Cats with benign hyperplasia usually have a more uniform distribution of cells or an inflammatory gradient that indicates the presence of an infectious agent or other cause of inflammation. The presence of the large cells with very prominent nucleoli in a background of small and intermediate lymphocytes with a sclerotic background gives this type of lymphoma a specific identity in both the cat and horse, and less commonly in dogs. Spreading by contiguity is only well defined in the cat and is not known to occur in dogs and horses.

Transformation and Prognosis

T-cell rich large B-cell lymphoma in cats is an indolent disease in which affected cats survive for several years and may succumb to other diseases. It appears that with progression the proportion of large cells increases. This change is generally not apparent unless serial biopsies are performed. The actual cytomorphology of individual cells does not appear to change with progression of the disease.

Treatment and Survival

T cell-rich large B cell type tumors respond poorly to aggressive therapy. This finding is logical due to the high level of connective tissue within the enlarged nodes. Animals tend to do best with conservative treatment involving brief cycles of aggressive therapy, recognizing that neoplastic B cells may be removed by this process without an appreciable decrease in node size.

Finally it should be recognized that there is not a defined difference between what has been called the Hodgkin's-like lymphoma in the cat and T cell-rich large B cell lymphoma. These appear to be one and the same disease and it is best termed T cell-rich large B cell lymphoma.

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T Cell Lymphoproliferative Diseases

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Application of the WHO Classification to T Cell Tumors in Domestic Animals

Precursor T Cell Neoplasms

T Cell Acute Lymphoblastic Leukemia

Lymphoblastic T Cell Lymphoma

Cranial mediastinal lymphoma

Mature T Cell Neoplasms

T Cell Large Granular Lymphocytic Leukemia

T Cell Chronic Lymphocytic Leukemia/

Prolymphocytic Leukemia and Small Lymphocytic Lymphoma

Enteropathy-type T Cell Lymphoma/Intestinal T Cell Lymphoma

Cutaneous T Cell Lymphoma

Mycosis fungoides

Pagetoid reticulosis

Non-epitheliotropic lymphoma

Sézary syndrome

T Zone Lymphoma

Extranodal NK/T Cell Lymphoma, Nasal Type

Hepatosplenic Gamma-Delta T Cell Lymphoma

Peripheral T Cell Lymphoma, Not Otherwise Specified

Natural Killer Cell Neoplasms

Unclassifiable, High-grade Plasmacytoid Type Lymphoma

Acronyms and Abbreviations

AITL, angioimmunoblastic T cell lymphoma; ALCL anaplastic large cell lymphoma; ALL, acute lymphoblastic leukemia; ATL, adult T cell lymphoma; BLV, bovine leukemia virus; CHOP, cyclophosphamide, vincristine, doxorubicin, prednisone; CLL, chronic lymphocytic leukemia; CTCL, cutaneous T cell lymphoma; EBV, Epstein Barr virus; ECL, epitheliotropic cutaneous lymphoma; ENKL, extranodal NK/T cell lymphoma; EORTC, European Organization for Treatment and Research of Cancer; ETTCL, enteropathy-type T cell lymphoma; FeLV, feline leukemia virus; FNA, fine needle aspirate; HTLV-I, human T lymphotropic virus-I; IBD, inflammatory bowel disease; IL, interleukin; LBTL, lymphoblastic T cell lymphoma; LGL, large granular lymphoma; LPD, lymphoproliferative diseases; MF, mycosis fungoides; NECL, non-epitheliotropic cutaneous lymphoma; NHL, Non-Hodgkin lymphoma; NK, natural killer; PTCL-NOS, peripheral T cell lymphoma, not otherwise specified; SLL, small lymphocytic lymphoma; SS, Sézary syndrome; TCR, T cell receptor; TPLL, T cell prolymphocytic leukemia; TZL, T zone lymphoma; WHO, World Health Organization.

Tcell lymphomas are a heterogeneous group of tumors whose common features include clonal rearrangements of T cell antigen receptor genes (TCR α , β , γ , or δ) and expression of CD3 complex proteins. In many cases, these tumors fail to express functional TCR, either because productive rearrangements do not occur in complementary matched genes that form TCR (α/β or γ/δ), or because one or more chains in the CD3 complex are absent. This prevents proper transport and assembly of the complex at the cell membrane. In most cases CD3 proteins will accumulate in the cytoplasm, and one or both of these findings (i.e. rearranged T cell receptor genes and accumulation of CD3 proteins) are useful for a preliminary classification of T cell lymphomas.

Compared to B cell lymphomas, relatively little information exists about the biology of T cell lymphomas. In people, these malignancies are estimated to account only for 12–15% of all lymphoproliferative diseases.^{7,154,186} However, there are geographical differences. T cell lymphomas are significantly more common in Asian populations, due at least in part to the endemic nature of human T lymphotropic virus-I (HTLV-I) and Epstein Barr virus (EBV), both of which are associated with specific T cell lymphoma subtypes.

T cell tumors also are incompletely understood in domestic animals. Recent efforts have allowed for classification of these tumors in the dog,²⁰⁰ and while most of these tumors are assumed to occur in other species,

peer reviewed reports are limited. The relative frequency and the overall incidence of T cell lymphomas in dogs might be higher than those observed in Western populations, although they may be lower than those observed in certain Asian countries such as Japan. Contemporary data suggest that T cell tumors account for approximately 30% of all lymphoproliferative malignancies in dogs.^{62,63,69,97,146,184,192} There appear to be heritable factors that predispose dogs to develop lymphoproliferative disorders in general. A high prevalence of T cell tumors in Boxers was found in several independent studies.^{97,121,146} Other breeds that are highly predisposed include Spitz breeds and small Asian lapdogs.^{97,133}

In cats, the relative frequency of T cell tumors seems to be in flux, evolving with the waning of the feline leukemia virus (FeLV) epidemic.¹²⁰ A heritable component also may exist in cats; several reports indicate that Siamese cats are predisposed to mediastinal lymphoma, a disease subtype that most commonly originates from malignant T cells.^{30,66,67,191} The relative frequency of T cell lymphoma in other domestic animals is unknown. While extranodal lymphomas, especially in the skin often originate from T cells, nodal T cell lymphomas also may be common in horses. In one study, 26 of 37 cases of equine lymphoma were of T cell origin and 25 had multicentric distribution with frequent mediastinal involvement.¹³⁰

APPLICATION OF THE WHO CLASSIFICATION TO T CELL TUMORS IN DOMESTIC ANIMALS

In 1994, the International Lymphoma Study Group proposed a revised World Health Organization (WHO) classification for human lymphoid neoplasms that combined morphologic, immunologic, and genetic assessments.⁸⁵ Modifications to the 2001 WHO classification scheme incorporated clinical features, further improving its prognostic and predictive accuracy (see Chapter 64).^{26,93}

The WHO classification scheme also was recently adapted to lymphoma in domestic animals,^{60,63,97,184,201,202} but studies are lacking to define the precise incidence and relative frequency of T cell lymphoma subtypes in these species. It is possible that this classification is artificial, as the concordance between the ontogeny and biology of lymphoma subtypes in humans and domestic animal has not been fully established. Indeed, the apparent high prevalence of T cell tumors in dogs might be due to differences in the underlying molecular mechanisms of lymphomagenesis between dogs and humans, although it also could reflect the relative importance of gene-environment interactions. For example, dogs are the only domestic species where neither a clinically important retrovirus nor a gamma-herpes virus has been identified. Nevertheless, the WHO system provides a useful scheme to guide comparative studies that will establish unique and shared properties of these

diseases in different species, and so improve our understanding of lymphomagenesis.¹⁶

Here, we describe T cell lymphomas according to the modified WHO classification^{200,201} with special emphasis on those tumors that are most relevant to domestic animals. Specifically, we will describe tumors derived from precursor T cells, including acute lymphoblastic leukemia (ALL) and lymphoblastic lymphoma, and tumors derived from mature cells, including large granular lymphoma (LGL), chronic lymphocytic leukemia (CLL), intestinal lymphoma, cutaneous lymphoma, T zone lymphoma (TZL), nasal lymphoma, hepatosplenic lymphoma, peripheral T cell lymphomas not otherwise specified (PTCL-NOS), natural killer (NK) cell tumors, and a recently described high-grade plasmacytoid type lymphoma. We refer the reader to other exceptional textbooks for a more complete description of rare T cell lymphomas in animals,²⁰⁰ and for a thorough overview of T cell malignancies in humans.^{47,49,103,188} We have chosen to retain TZL as a specific descriptor because this condition is common in at least some breeds of dogs,^{60,200} and we included NK cell tumors in a separate category, owing to the scarcity of reagents to identify these cells in domestic animals and the consequent paucity of information.

PRECURSOR T CELL NEOPLASMS

Both T cell ALL and lymphoblastic T cell lymphoma (LBTL) are included in the precursor T cell neoplasm category. The considerable overlap of morphologic, clinical and immunologic features between these diseases has led some to suggest that these two conditions may represent different manifestations of the same disease.¹⁹⁰ Presently, the classification is largely based on where the largest burden of disease is present (blood or bone marrow for ALL and solid tissues for LBTL).

T Cell Acute Lymphoblastic Leukemia

As is true for most other acute leukemias, T cell ALL cannot be diagnosed based on cellular morphology alone. Instead immunophenotyping and assays to establish the presence of a clonal TCR rearrangement are needed to reach this diagnosis (see Chapter 142). Surface CD3 expression can be absent, making detection of cytoplasmic CD3 necessary for accurate classification. Most acute leukemias express CD34,^{206,216} but there are few published studies examining the distribution of this antigen on different leukemic subtypes. Efforts to define phenotypic subtypes of acute leukemia in domestic animals are mostly restricted to dogs. Among ALL cases, the observed frequency of T cell leukemias in dogs seems to mirror the overall frequency of all T cell tumors.^{166,197,205}

Various recurrent genetic abnormalities have been defined in humans that probably contribute to the pathogenesis of T cell ALL, including translocations and fusions that juxtapose transcription factors with promoters or enhancers within the TCR, aberrant expression of

genes that disrupt thymocyte development, inactivation of cell cycle regulatory genes, and constitutive activation of non-receptor protein tyrosine kinases that provide a survival advantage for immature T cells.⁴⁴

The clinical features of T cell ALL are largely related to infiltrative bone marrow disease, which causes peripheral cytopenias that lead to fever, increased risk for infection, and bleeding tendencies. However, ALL cells can also infiltrate visceral organs, disrupting architecture and leading to severe dysfunction or failure (see Chapter 65).²¹⁴ While chemotherapy protocols have improved survival of some types of B cell ALL in people, especially children, the same cannot be said for T cell ALL where 5 year survival rates remain below 60%.^{6,28} Cell counts and expression of various maturation-associated antigens can be prognostic in people. A threshold of >100,000 cells/ μ L at presentation is associated with inferior survival,⁸⁸ and early precursor phenotypes are indicators of poor prognosis.^{31,35}

Lymphoblastic T Cell Lymphoma

Lymphoblastic T cell lymphomas are highly aggressive tumors of immature T cells that are seen more commonly in children and young adults. Almost all lymphoblastic lymphomas in humans are LBTL.⁷⁵ Anterior (cranial) mediastinal lymphoma appears to be a relatively common, unique entity within the T cell ALL/LBTL complex.

Lymphoblastic T cell lymphoma originates from a precursor cell and can, therefore, have CD4/CD8 double negative or double positive phenotypes.^{63,84} Similar to T cell ALL, surface CD3 expression can be absent, making the detection of cytoplasmic CD3 necessary. The tumors show diffuse effacement of nodal architecture, invasion into the subcapsular sinus and often extension into perinodal tissues by a cytologically bland population (Fig. 72.1). Despite their description as blasts, cells are of intermediate size, have round to indented euchromatic nuclei with indistinct nucleoli, and scant to modest amounts of lightly basophilic cytoplasm (Fig. 72.2). Unlike low-grade T cell lymphomas, these tumors can exhibit high mitotic activity and prominent staining with proliferation-specific markers such as Ki-67.

A recent study showed that the CDKN2 locus that encodes the cyclin kinase inhibitors p16 and p15, and the p53 regulator p14/ARF, was deleted in 10/10 high-grade canine T cell lymphomas, including 7/7 LBTL.⁶⁰ In contrast, this abnormality was not present in any of 11 low-grade T cell lymphomas (T zone lymphoma [TZL] or small lymphocytic lymphomas) or 26 high-grade and low-grade B cell lymphomas. These tumors also are genetically unstable, harboring numerous additional cytogenetic abnormalities.^{110,193} The mechanisms that account for genetic instability in these tumors are unknown, but recent gene expression profiling experiments suggest canine LBTL resembles other high-grade tumors, showing enrichment of genes that control metabolism, proliferation, and survival at the expense of genes that comprise functional ontogenetic pathways (A. Frantz, T.L. Phang, J.F. Modiano, unpublished data).

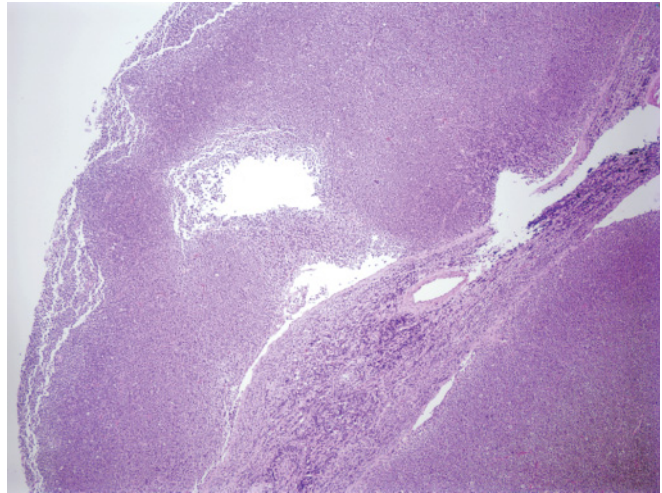


FIGURE 72.1 Lymphoblastic T cell lymphoma in a lymph node from a dog. Notice diffuse effacement of nodal architecture and invasion into the subcapsular sinus by a monomorphic population of lymphocytes. H&E stain; original magnification 40 \times .

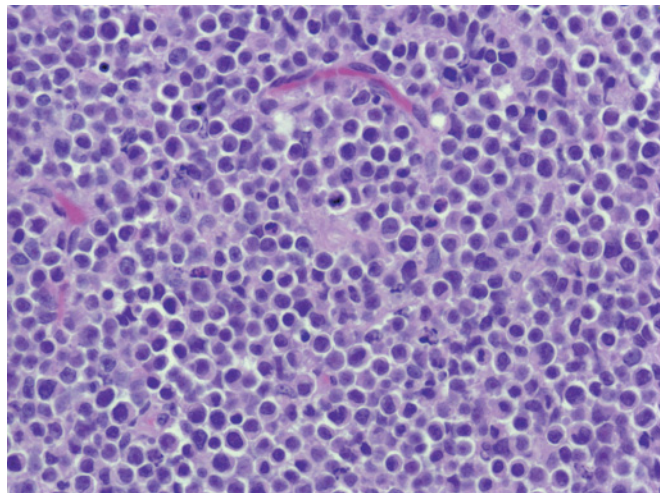


FIGURE 72.2 Lymphoblastic T cell lymphoma in a lymph node from a dog. Notice pleomorphic cells, generally of intermediate size with indented euchromatic nuclei, and modest amounts of lightly basophilic cytoplasm. In this case, nucleoli are apparent in many cells, a rare feature in these tumors. H&E stain; original magnification 500 \times .

Therapy for LBTL in dogs is generally unrewarding unless it is confined to the mediastinal space. The median survival with cyclophosphamide, vincristine, doxorubicin, prednisone (CHOP)-based chemotherapy for the dogs described above with LBTL and CDKN2 deletion was less than 4 months, and none of the dogs survived 1 year.⁶⁰

Cranial Mediastinal Lymphoma

In humans, LBTL of the mediastinum is a defined subtype of precursor T cell lymphoma that usually

presents as an anterior mediastinal mass with or without blood and bone marrow involvement.^{53,117} In domestic animals, primary mediastinal lymphoma (also known as thymic lymphoma) has been reported in the dog, cat, horse, cow, ferret, and pig, most commonly as a T cell disease,^{29,63,66,99,112,113,140,141,217} although this diagnosis may include a more heterogeneous group of diseases.

Incidence data are not published in all species, but cranial mediastinal lymphoma is considered, respectively, as the most common cause of thymic pathology in the cat and the second most common cause of thymic pathology in the dog.⁴¹ This is also the most common thoracic neoplasm in the horse.^{122,187} There may be heritable predisposition in the Boxer breed,¹²¹ in Siamese cats (specifically as a form of juvenile thymic lymphoma not associated with FeLV infection),^{67,120} and perhaps in golden retrievers.

Most cases of mediastinal LBTL in humans occur in adolescents and young males, and they are the most common mediastinal tumors in children.^{8,185,199} Mediastinal lymphoma does not have an age predilection in dogs,⁴¹ but it is most commonly seen in younger cats.^{41,120} In cows, mediastinal LBTL is one of three forms of sporadic bovine lymphoma (not associated with BLV infection), and is most common in animals between 6 months and 2 years of age.²¹⁷

As is noted for other peripheral lymphomas outside the gut, primary mediastinal lymphoma has become less common in cats as the prevalence of FeLV infection has decreased. Prior to 1980, mediastinal lymphoma was reported to account for up to 40% of feline lymphoma in the United States, 50% in the UK, and 70% in Japan.^{33,81,177,189} More recently, mediastinal lymphoma was reported to represent less than 12% of cases in the United States and approximately 25% of cases in Australia.^{30,67,120,198}

In people, there can be immunophenotypic overlap between cells from LBTL and cells from other non-lymphomatous lesions in the mediastinum, most notably those from cases of thymoma and thymic hyperplasia. As such, a diagnosis of LBTL requires the identification of a thymocyte-like (i.e. CD4/CD8-double positive) population demonstrating loss of one or more of the normal thymocyte cell surface antigens (CD2, CD3, CD5, CD7, and/or CD45), disproportionately high numbers of CD4/CD8-double negative, CD4-single positive, or CD8-single positive cells, or expression of CD34 by an atypical population of mature thymocytes.^{78,117} Flow cytometric light scatter also can help distinguish neoplastic LBTL cells from normal thymic components.^{78,117}

The diagnostic challenge of mediastinal LBTL is magnified in animals. Imaging alone is insufficient to distinguish lymphoma from other mediastinal lesions such as thymoma, carcinoma, chemodectoma, and sarcoma.^{157,218} Fine needle aspirate (FNA) cytology is useful as an adjunct to diagnosis, but distinguishing thymic lymphoma from thymoma in FNA samples from dogs is difficult because of the large number of non-neoplastic lymphocytes present in the latter.^{9,112}

Lymphoblastic T cell lymphoma cells in mediastinal lymphomas of domestic animals can also show phenotypic heterogeneity, although they frequently have a single positive CD3, CD4 phenotype in dogs.^{63,112,121,134} In cats, the majority of cases of feline anterior mediastinal lymphoma consist of CD3, CD5, CD8-positive T cells.^{66,116,148,177,198} In cows, anterior mediastinal lymphoma is a CD2, CD3, and/or CD5-positive T cell disease, most commonly of CD4/CD8-double negative cells.^{4,169,217} Similar characterization is lacking in other species.

MATURE T CELL NEOPLASMS

T cell Large Granular Lymphocytic Leukemia

T cell large granular lymphocytic (LGL) leukemias account for 2–3% of small lymphocytic leukemias in people.¹⁴⁴ It is typically a disease of older adults (median age 61 years) with equal gender distribution.⁵⁰ Autoimmune diseases including rheumatoid arthritis and Sjogren's syndrome, and detection of anti-nuclear antibodies are reported as common co-morbidities with T-cell LGL leukemia.^{50,144} Anti-platelet antibodies, anti-neutrophil antibodies, and positive Coombs' tests have been reported in subsets of people with LGL leukemia.⁵⁰ T cell LGL leukemia is typically an indolent disease in people, and a significant proportion of affected patients do not require therapy at diagnosis. With treatment, median survivals of 161 months have been reported.⁵⁰

T cell LGL leukemia has been reported in the dog, cat, horse, and cow, with much of the published literature focusing on the first two species.^{107,109,163,167} Large granular lymphocytic leukemia of the dog and cat is most commonly a T cell disease, as more than 90% of cells in lesions from each are of CD3-positive T cell origin.¹⁶³ In the dog, T cell LGL leukemia is reported to be a common form of CLL, representing 54% (39/73) of cases in one study. It is a clinically heterogeneous disease in dogs with a broad spectrum of presentations and outcomes.¹²⁶ In canine LGL leukemia, neoplastic cells are commonly seen in the blood.¹²⁶ Infection with *Ehrlichia canis* needs to be excluded as it can result in a selective increase in circulating LGLs.²¹⁰ In contrast, feline LGL malignancies are typically aggressive. Gastrointestinal tract-associated conditions are frequent with fewer than 10% of cats presenting with a leukemic component.¹⁰⁹ Median survival time in treated cats has been reported to be 57 days.¹⁰⁹

Clinical features in the dog are variable; splenomegaly (12 of 20 dogs) is reported as the most common physical examination finding.¹²⁷ In contrast, feline LGL is typically characterized by anorexia, lethargy, weight loss, and vomiting, with an abdominal mass, described in 52% of cases, reported as the most common physical examination finding.^{109,163}

A diagnosis of LGL leukemia in the dog and cat is generally made through the cytologic detection of the characteristic neoplastic cell in blood or tissue. These cells are typically large (10–35 μm) and pleomorphic

with nuclear variability and a variable number of irregularly shaped and sized magenta, intracytoplasmic granules. The majority of feline cases of LGL disease are composed of CD18 (21/21), CD3 (19/21), homodimeric CD8 (13/21), and CD103 (11/19)-positive cells, consistent with intestinal origin.¹⁶³ Rare, CD4 single positive, and CD4, CD8 double-positive cases have been reported.¹⁶³ In the dog, T cell LGL is described as a phenotypically heterogeneous disease in which CD3, leukointegrin $\alpha_4\beta_2$ -positive cells predominate (>90% of cases), with variable TCR expression. The leukointegrin expression has been proposed to indicate that canine LGL leukemia originates in the splenic red pulp with bone marrow involvement only seen in the advanced stages.

An etiology has not been demonstrated for domestic animal T cell LGL leukemia, as the vast majority of affected cats are negative for FeLV and FIV infection.^{109,163} In dogs, a single report describes the presence of retroviral particles, although the significance of this remains uncertain.⁷²

T Cell Chronic Lymphocytic Leukemia/ Prolymphocytic Leukemia and Small Lymphocytic Lymphoma

In the late 1980s, the French-American-British (FAB) classification system subdivided mature T cell leukemias into four categories; T cell chronic lymphocytic leukemia (CLL); T cell prolymphocytic leukemia; human T lymphotropic virus type 1-positive (HTLV-1) adult T cell leukemia/lymphoma; and Sézary syndrome.¹³ The original category of T cell CLL was updated in 2001 to T cell prolymphocytic leukemia (TPLL) where leukemias with small, mature-appearing lymphocytes are now considered as a small cell variant of prolymphocytic leukemia. There is no consensus for the use of prolymphocytic leukemia versus CLL in domestic animals, and CLL is used mostly by convention to describe an expanded population of small lymphocytes in the circulation. When the disease is confined to lymphoid organs, it is called small lymphocytic lymphoma (SLL).

The characteristic cell in human TPLL is described as a small to medium sized prolymphocyte with a prominent single nucleolus and basophilic cytoplasm. In domestic animals, T cell CLL is characterized by small to medium sized cells with round to oval nuclei, smooth chromatin, and scant to moderate clear to lightly basophilic cytoplasm that may contain azurophilic granules (Fig. 72.3).

In humans, the median age at diagnosis of TPLL is 63 years.⁴⁶ In contrast to the more common B cell CLL, TPLL commonly presents with peripheral lymphadenopathy, splenomegaly, and peripheral lymphocyte counts greater than 10,000/ μ L; a subset of patients will present with cutaneous involvement, particularly of the head and neck.^{34,125} In animals, T cell CLL manifests as an indolent disease that is often diagnosed incidentally. Unlike acute leukemias, bone marrow infiltration is

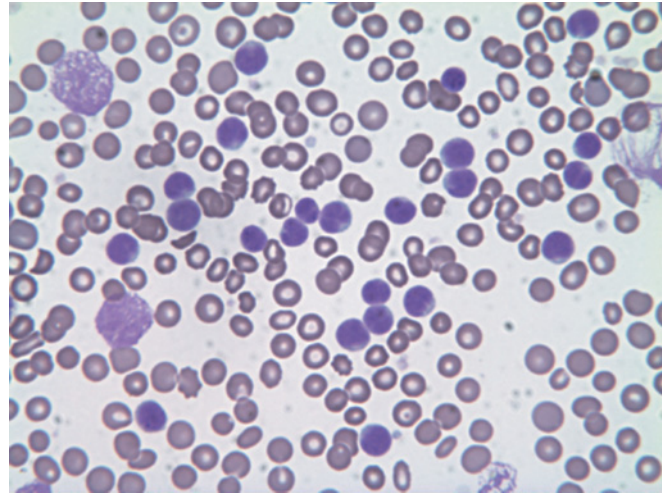


FIGURE 72.3 Chronic lymphocytic leukemia (CLL) in a blood smear from a dog. Notice an expanded population of small lymphocytes characterized by round to convoluted nuclei without nucleoli and scant light cytoplasm. In this case, cells are agranular, but azurophilic granules are commonly seen in CLL cells. Diff-Quik stain; original magnification $\times 560$.

inconsistent and peripheral cytopenias are rare, although splenomegaly and lymphadenopathy are reported in up to 30% of affected dogs.^{115,126,213}

The phenotypic characteristics of TPLL in humans and T cell CLL in dogs are consistent with post-thymic origin. In humans, TPLL is most often associated with a helper/inducer phenotype (CD4),⁴⁶ and while it is possible some of these tumors arise from CD4/CD25/FoxP3 regulatory T cells, this manifestation may be more common in the Sézary syndrome (see below). In dogs and cats, CLL is most often a disease of T cells, including large granular lymphocytes.^{197,205,216} Canine T cell CLL usually involves expansion of a CD8 clone, whereas in cats and horses, CLL often comprises CD4 T cells.^{37,130,158,216}

A peculiar feature of TPLL in humans and CLL in dogs and cats, is aberrant expression of cell surface markers.⁹⁴ For example, in a study of 87 T cell tumors, Gorczyca et al. found that complete loss of any T cell antigen (CD2, CD5, CD7) or the pan-leukocyte antigen CD45 was diagnostic for malignancy.⁷⁹ Loss of CD45 appears to be the most common form of aberrant antigen expression in canine T cell CLL, and loss of CD4 and CD8 also may occur in some cases of canine and feline T cell CLL (Anne Avery, personal communication).^{205,213}

Management strategies for CLL are discussed in Chapter 63. Recent data suggest that the phenotype and number of circulating cells are prognostic in dogs. In one study, dogs with less than 30,000 lymphocytes/ μ L in the peripheral circulation had significantly longer survival (mean = 1,098 days) than dogs with more than 30,000 lymphocytes/ μ L (mean = 131 days).²¹³

Enteropathy-type T Cell Lymphoma/Intestinal T Cell Lymphoma

Enteropathy-type T cell lymphoma (ETTCL) is a relatively rare condition that accounts for approximately 5–10% of NHL and 1–4% of all gastrointestinal tumors in people.⁵¹ In 2001, the WHO classification of intestinal T cell lymphoma was modified to include ETTCL because it was deemed more specific, although ETTCL can occasionally arise from extra-intestinal sites.²⁶ O'Farrelly originally coined the term enteropathy-associated T-cell lymphoma to denote the association of this lymphoma with jejunal mucosal atrophy.¹⁴⁴ Enteropathy-type T cell lymphoma is now recognized as a tumor that affects the gastrointestinal tract causing villous atrophy and crypt hyperplasia, with involvement of the regional lymph nodes, and potentially the spleen and liver. The small intestine is the primary affected site, but this form of lymphoma also can involve stomach and colon. Males tend to be overrepresented, and the disease has a worse prognosis than intestinal lymphomas arising from B cells.¹⁰⁴

Most ETTCLs are believed to originate from intraepithelial CD8 (CD103-negative) T cells with large anaplastic morphology; however, about 20% arise from small, atypical, cytotoxic lymphocytes or NK T cells that express CD3, CD8, CD56, and CD103, and that do not cause enteropathy-related lesions.²¹⁹ While these tumors may have distinct clinicopathologic features,¹³⁹ they are both characterized by aggressive biological behavior.³

Enteropathy-type T cell lymphoma has not been described as a specific entity in animals, although intestinal T cell lymphoma is relatively common in both epitheliotropic (lymphocytes infiltrate the epithelial layer) and non-epitheliotropic (lymphocytes do not cross the basement membrane) forms that also can involve one or more areas in the gut, regional lymph nodes, spleen, and liver. In the dog, intestinal T cell lymphoma accounts for more than 70% of intestinal lymphoid tumors and is frequently epitheliotropic, but the immunophenotype of these tumors (T cell versus B cell) was not prognostically significant in the small number of cases examined to date.⁶⁴

In cats, the reduced incidence of retroviral-induced lymphomas has led to an apparent increase in the frequency of alimentary lymphoma. Almost 40% of feline lymphomas from a retrospective study spanning 20 years affected the gastrointestinal tract, and the authors noted an increase in the incidence of intestinal lymphoma in the last 10 years of the study.¹²⁰ However, these data should be interpreted cautiously given the limitations of case-control and retrospective studies. The majority of alimentary lymphomas in cats have been described as having a B cell phenotype; yet, recent reports indicate tumors likely to have originated in the small intestine are more commonly T cell lymphomas.^{66,91,147,149,208} Epitheliotropism is seen in most low-grade feline tumors.^{24,108} When cats with increased large granular lymphocytes in the circulation or in aspirates of abdominal organs were examined, they uniformly

had T cell intestinal lymphoma arising from CD8-single positive cells, consistent with their putative intraepithelial lymphocyte origin.¹⁶²

The pathogenesis of ETTCL in humans and intestinal T cell lymphoma in animals is incompletely understood. There seems to be a spectrum of infiltrative, lymphoplasmacytic infiltrative lesions that range from mild inflammation with a diagnosis of inflammatory bowel disease (IBD) to effacement of intestinal architecture by monomorphic populations that are undoubtedly lymphoma. There may be a progression from antigen driven lymphocytic inflammation to lymphoma. For example, 5–10% of patients with an inflammatory enteropathy called celiac disease (“gluten allergy”) are refractory to gluten withdrawal therapy, and patients with refractory celiac disease are at high risk to develop intestinal T cell lymphoma, suggesting these tumors may originate from intraepithelial lymphocytes expanded as part of the inflammatory response.²⁰⁴ A similar progression of IBD to intestinal lymphoma has been hypothesized to occur in cats.^{24,155} For example, one cat diagnosed with alimentary lymphoma survived 28 months with conservative management using prednisone and a novel protein diet.²⁴

Clinical signs associated with ETTCL are referable to gastrointestinal dysfunction and depend on the anatomical site and extent of infiltration. Although morphologically the cells are “bland” in appearance, the disease is highly aggressive in people, with one study reporting less than 20% 5 year survival and another study reporting less than 30% 2 year survival.^{38,68} Clinical signs of intestinal lymphoma in animals also are referable to the anatomic site and extent of infiltration. Paraneoplastic eosinophilia and eosinophil infiltration of the tumor have been reported in canine, feline, and equine T cell intestinal lymphoma. In humans, eosinophil production and recruitment are associated with tumor production of interleukin-5 (IL-5),^{11,18} but such association remains to be documented in veterinary species.

Diagnosis of ETTCL or intestinal lymphoma requires complementary use of clinical signs, imaging, and microscopic pathology. Aspiration cytology of thickened bowel or enlarged mesenteric lymph nodes often can lead to a diagnosis if a uniform, atypical population of lymphocytes is seen. The cytomorphology of human ETTCL is highly variable. The cells are generally medium-sized to large but in a subset of cases, they can be anaplastic and express surface CD30, a characteristic of anaplastic large cell lymphoma (ALCL).⁵⁶

Morphologic distinction between lymphocytic inflammation and lymphoma of the gut can be challenging, underscoring the utility of immunophenotyping and clonality assays.^{39,208} High-grade alimentary lymphomas are poorly responsive to therapy, especially because gastrointestinal signs can be intractable. Indolent intestinal T cell lymphomas in dogs and cats can be successfully managed with low-intensity chemotherapy, but lack of prognostic indicators makes this disease a therapeutic challenge.

Cutaneous T Cell Lymphoma

Cutaneous lymphoma (LPD) are relatively uncommon peripheral neoplastic diseases that are, in both humans and veterinary patients, characterized by a widely heterogeneous clinical presentation. Cutaneous lymphomas represent 1% and 2.8% of all canine and feline skin tumors, respectively.⁷⁶ Cutaneous lymphoma is predominantly a disease of older animals, with a mean age of 9–12 years in the dog, 8–17 years in the cat, and 3–19 years in the horse.^{40,43,54,70,87,136,137,153,173,195,215} In cattle, cutaneous lymphoma most commonly develops in animals between 2 and 3 years of age. In no species has a sex predilection been identified.

In veterinary medicine, cutaneous LPD is most commonly subdivided into epitheliotropic and non-epitheliotropic forms, based on the presence or absence of invasion of the epithelium of the epidermis and/or adnexae.^{129,172} In contrast, cutaneous lymphoma in humans comprises a broader spectrum of conditions including T cell, NK cell, and B cell neoplasms as well as immature hematopoietic malignancies and Hodgkin's lymphomas.²¹ The WHO classification scheme considers these as independent entities, and the presence or absence of epitheliotropism is only one part of a multifaceted diagnostic algorithm that includes clinical presentation, histology, immunophenotyping, and molecular analysis.²¹

Despite these classification differences, there are many similarities between the cutaneous LPDs of humans and domestic animals. Most notably is the observation that most arise from T cells, and are therefore denoted as cutaneous T-cell lymphomas (CTCLs).^{40,137} Here, we have used the WHO classification scheme as a framework to organize the descriptive properties of CTCL in animals, recognizing that not all of the current veterinary literature fits neatly into this scheme. Those entities within veterinary medicine with WHO counterparts are: mycosis fungoides (MF), pagetoid reticulosis, and Sézary syndrome (SS).

Mycosis Fungoides

Mycosis fungoides (MF), in both humans and most domestic species, is the most common form of CTCL.^{19,21,40,137} It is characterized by invasion and effacement of the epidermal and/or adnexal epithelium by neoplastic cells, a property which has led to its more descriptive designation, epitheliotropic cutaneous lymphoma (ECL). Mycosis fungoides is reported in dogs, cats, horses, ferrets, cows, hamsters, squirrels, and a coati.^{87,89,129,137,164,171,175,180,195} It is the most common cutaneous LPD in the dog and cow.^{19,40} Briards, English cocker spaniels, bulldogs, Scottish terriers, and golden retrievers may be predisposed to develop cutaneous lymphoma.⁷⁶

In both humans and domestic animals, an underlying etiology for MF remains to be identified. In humans, retroviruses, environmental exposures, genetic mutations and infection with *Staphylococcus aureus* and

Chlamydia spp. have been implicated, but studies have yielded inconsistent results.^{1,73,90} In the cat, attempts to link retroviral infection to MF have been unsuccessful.^{23,57,136,142,173,195} An infectious cause of MF also has not been identified in dogs, although retroviral particles have been described in a cell line.⁷¹ In the cow, the majority of cases of MF are manifestations of sporadic bovine lymphoma, which is not associated with BLV infection.

In addition to infectious, environmental, and genetic causes, a link between atopic dermatitis and MF has been proposed in humans based on the common phenotype of the infiltrating lymphocytes in both diseases (CD3/CD4/CD45RO-TCR $\alpha\beta$ -positive and CD8/CD30-negative memory T cells), an overlap in their cytokine profiles, and increased circulating levels of IgE in patients with MF.^{127,156,174} Although the results of such human studies are mixed, work in dogs supports a similar hypothesis. In addition to the phenotypic similarities between the lymphoid population in canine atopic dermatitis and MF, canine MF predominantly develops in anatomic areas affected with allergic disease.⁵² Moreover, a retrospective study of canine MF and atopic dermatitis suggests that there is an incidence association between the two diseases.^{127,168} The clinical presentation of MF is heterogeneous, and has been described in detail elsewhere.^{80,176} Generally, it involves an exfoliative dermatitis that is refractory to therapy. In humans, MF is most commonly a disease of mature, memory helper T cells (a CD2, CD3, CD4, TCR $\alpha\beta$, CD5, CD45RO-positive phenotype), while only rare cases of CD8 and TCR $\alpha\beta$ MF have been reported.²¹ Although particularly aggressive cases might originate from regulatory T cells.⁸³ In contrast, MF in dogs is predominantly a disease of cytotoxic CD8, TCR $\alpha\beta$ -positive T cells, with few CD4-positive or CD4/CD8-double negative cases reported.^{58,137} A high-grade variant of CTCL in humans originates from anaplastic large cells that express CD30. The authors have yet to encounter a homologous CD30-positive T cell-derived ALCL but the morphological features of cutaneous ALCL have been described.²⁰⁰ Intriguingly, the majority of MF cases in the dog do not express CD5.¹³⁵

Information about feline MF is limited, but this also appears to be primarily a disease of CD3-positive T cells.^{22,23,96,142,173,195} Expression of perforin, a cytotoxic protein stored in the cytoplasm of cytotoxic T cells and NK cells, was documented in one case,^{118,142} but others state that CD4-, CD8-double negative cells predominate in this disease.⁸⁰ Mycosis fungoides in horses and cows is also poorly defined.^{1,19,36,87,100,153,170,181}

Skin biopsy with histopathology is required to diagnose MF in people and domestic animals, although neoplastic lymphoid cells that merit including MF in a differential list can be identified in fine needle aspiration samples. The pathognomonic features of MF include the presence of a population of neoplastic round cells demonstrating selective tropism for the epidermal, mucosal, and/or adnexal epithelium. Additionally, small aggregates of intraepithelial lymphocytes (i.e.

Pautrier's microabscesses) can occasionally be identified throughout the epidermis.⁸⁰ In early lesions, light microscopic diagnosis may be difficult due to the presence of an infiltrate that contains relatively few overt neoplastic cells. As the disease progresses, the cellular infiltrate becomes more homogeneous, with small cells that resemble those seen in T zone lymphomas. Adjunctive diagnostic modalities such as immunohistochemistry and molecular analysis may be necessary to diagnose MF.⁵⁹

Pagetoid Reticulosis

Pagetoid reticulosis is a low-grade variant of MF with characteristic histomorphologic appearance.²¹ Microscopically, pagetoid reticulosis is characterized by prominent epitheliotropism of vacuolated neoplastic cells into a thickened epithelium.²¹ In contrast to MF, pagetoid reticulosis has been described as a form of epitheliotropic lymphoma in which the neoplastic population is confined almost completely to the epidermal layer and in which the underlying dermis is infiltrated by a pleocellular, inflammatory infiltrate.⁸⁰ A single case of pagetoid reticulosis is reported in a dog.⁹⁵

Non-epitheliotropic Lymphoma

In the WHO classification, non-epitheliotropic cutaneous lymphoma (NECL) is not recognized as a specific entity, but rather, the lack of significant epidermal invasion is a histologic feature of various diseases such as subcutaneous panniculitis-like T cell lymphoma and other forms of primary, cutaneous T cell lymphoma.²¹

NECL has been reported in dogs, cats, horses, and cows, and it appears to be the least common form of cutaneous lymphoma in the dog and the most common form in the cat and cow.^{43,48,77,106,159,178,196} The etiology of NECL is unknown,^{23,57} and the clinical features resemble other types of CTCL. The cellular phenotypes in NECL also are similar to those of MF and other CTCL, consisting primarily of CD3-positive T cells, which in the case of the dog can be CD8-single positive or CD4/CD8-double negative cells.^{40,136} In the cat, most published cases described CD3-positive neoplastic cells.^{40,106} In the cow and horse, limited data also indicate that NECL is a T cell neoplasm.^{19,43,175}

Microscopically, this disease is characterized by infiltration of the dermis and subcutis by a population of neoplastic round cells with relative sparing of the epithelium of the epidermal and adnexal structures.⁸⁰ As is true for ECL, histopathology is necessary to demonstrate the presence or absence of epitheliotropism.⁹

Sézary Syndrome

In the WHO-European Organization for Treatment and Research of Cancer (EORTC) classification scheme, Sézary syndrome (SS) is described as the leukemic form of CTCL.²¹ The disease is characterized by the presence of neoplastic T cells (Sézary cells) in the blood and

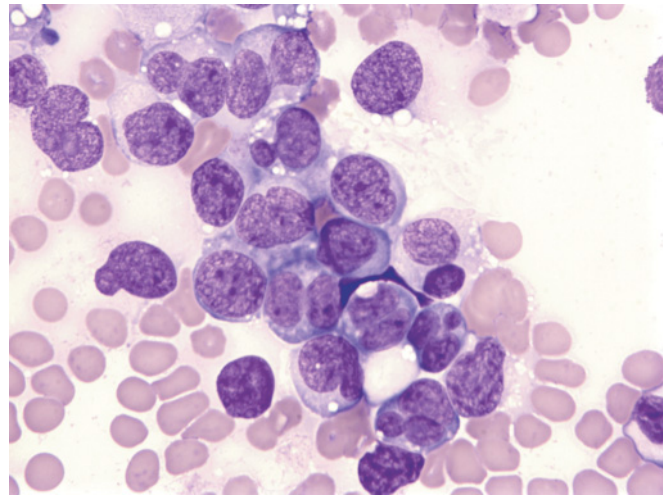


FIGURE 72.4 Sézary cells in a blood smear from a dog. Notice a population of pleomorphic lymphocytes characterized by round, lobulated, or complex nuclei with coarsely stippled to reticular chromatin and moderate amounts of vacuolated, lightly basophilic cytoplasm. Sézary cells in blood tend to show significantly greater pleomorphism than MF cells in the skin. Diff-Quik stain; original magnification $\times 500$.

lymph nodes (Fig. 72.4). It is a very rare manifestation of CTCL and, in domestic animals, has been reported in dogs, cats, and horses.^{16,150,160,173,194,215}

The clinical presentation of human SS includes generalized reddening and scaling of the skin (erythroderma) with lymphadenopathy. In animals, SS causes exfoliative, erythematous dermatitis that can cause severe pruritus and lymphadenopathy.^{61,80,173,215} In humans, the cellular infiltrate arises from mature helper T cells with the same phenotype reported for MF.²¹ There is limited information on the immunophenotype in animals, but two reports in dogs suggest either a prototypical MF phenotype (i.e. CD3, CD8-positive) or NK-like phenotype (CD3, CD4, CD8, TCR-negative).^{71,215} In cats, there is a single case report where cells in the circulation were CD3+, CD8+, CD4- T cells.²¹⁵

In humans, the International Society for Cutaneous Lymphoma criteria for a diagnosis of SS are: an absolute Sézary cell count of at least 1,000 cells/ μL ; an expanded CD4+ T cell count resulting in a CD4/CD8 ratio of more than 10 where the cells may lack expression of CD2, CD3, CD4, or CD5; or the demonstration of a T cell clone in the blood by molecular or cytogenetic studies.²⁰⁹ Explicit criteria have yet to be established in veterinary hematopathology; thus, a diagnosis of SS in domestic animals relies on the identification of: (1) cutaneous lesions consistent with MF, and (2) circulating neoplastic T cells.

T Zone Lymphoma

T zone lymphoma as a distinct disease entity was introduced in the Kiel classification of 1974, but abandoned in the most recent WHO classifications because of

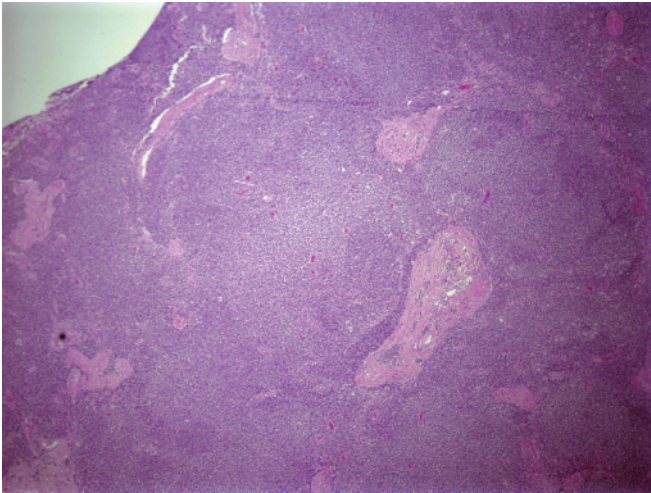


FIGURE 72.5 T-zone lymphoma in a lymph node from dog. Notice an expansion of lymphocytes in the parafollicular zone, residual follicular structures, and compression of the peripheral sinus. Also notice the prominent small vessels interlaced within the tumor. H&E stain; original magnification $\times 40$.

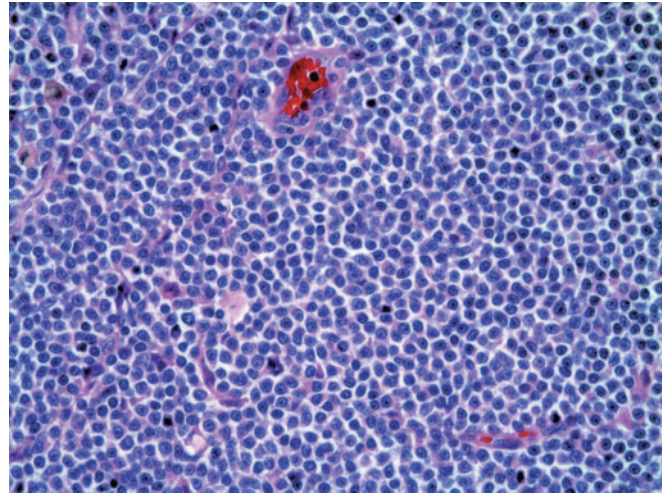


FIGURE 72.6 T-zone lymphoma in a lymph node from a dog. Notice the typical monomorphic appearance of the cells, which are small in size, have round nuclei with occasional indentations, smooth to stippled chromatin, prominent chromocenters, no apparent nucleoli, and modest amphophilic cytoplasm. Also notice the low mitotic activity and prominent association with a vascular network, suggestive of angioimmunoblastic lymphoma. H&E stain; original magnification $\times 500$.

significant morphologic and biologic overlap with angioimmunoblastic T cell lymphomas (AITL) and other indolent T cell lymphomas. Retaining this category in the classification of domestic animal lymphomas appears to have merit in the case of the dog, as TZL may be the most common indolent lymphoma at least in some breeds.^{60,200} The disease is characterized by expansion of small to intermediate lymphocytes in the parafollicular zone, leading to follicular collapse (i.e. fading follicles), compression of the peripheral sinus and some degree of medullary sclerosis (Fig. 72.5). In contrast to indolent B cell lymphomas arising from the marginal zone, the cellular population responsible for TZL is eccentric to the follicles, rather than surrounding them. The main distinction between the TZL and AITL is the prominent vascular component in AITL, although these two diagnoses may represent a spectrum of the same disease. Morphologically, TZL cells have few distinguishing characteristics: they tend to have nuclear clefts or indentations, smooth to stippled chromatin that may form prominent chromocenters, indistinct nucleoli, scant, clear to amphophilic cytoplasm, and low mitotic activity (Fig. 72.6).

T zone lymphomas generally have a helper (CD4) phenotype and seem to display slow clinical progression, but controlled studies are lacking to determine the most appropriate therapy for this disease. It is clear that TZL does not have the same poor duration of response and short patient survival commonly associated with high-grade T cell lymphoma. In fact, a recent study showed that, among 40 canine lymphomas, these tumors had by far the best response to standard care.⁶⁰ Furthermore, these tumors may respond even better to conservative management with low intensity chemotherapy, although data to support this are only anecdotal.

Extranodal NK/T Cell Lymphoma, Nasal Type

Extranodal NK/T cell lymphoma (ENKL) is a rare form of human lymphoma, although the incidence is higher in East Asia and Latin America where the majority of cases are associated with EBV infection.¹⁸⁶ Most cases of ENKL in humans arise from NK cells,¹¹⁸ although both the NK cell and T cell variants are considered as a single disease entity that share clinical and prognostic features.

Nasal lymphoma is rare in dogs, but it is the most common tumor of the nasal cavity in cats, with increased incidence since the inception of routine FeLV vaccination.^{5,86,120,131,138} Common clinical signs in dogs and cats include sneezing, upper respiratory stridor, and nasal discharge that can be purulent, serous, and bloody; facial pain and/or deformity are rare.^{109,196} The association of nasal lymphoma with systemic lymphadenopathy or spread to other distant sites is unclear. A recent publication reported 10 of 22 cats with multi-organ involvement of brain, gut, liver, lung, kidney, eye, and bone marrow.¹⁰⁹

Two independent case reports in dogs supported T cell ontogeny.^{102,196} Nasal lymphoma in the cat is predominantly a B cell tumor (70–90% of the cases).^{42,109,138}

Histology is preferred for diagnosis of nasal lymphoma, but brush cytology of the nasal passages can provide diagnostic samples if there is minimal inflammation and hemorrhage.¹⁰⁹ Squash preparations from endoscopic biopsies are also a reliable source of tissue for cytologic assessment, although it can be difficult to distinguish between lymphoma and lymphoid hyperplasia.⁴⁵

Hepatosplenic Gamma-Delta T Cell Lymphoma

In humans, hepatosplenic T cell lymphoma (HSTCL) is a clinically aggressive neoplasm that was first described as an entity in 1981.⁹⁸ It is characterized by hepatosplenic involvement, sinusoidal tropism, and an infiltrating population of $\alpha\beta$ TCR-positive T cells that also express CD2, CD3, and CD56.^{93,122,165,183,203} Although selected cases of TCR $\alpha\beta$ have been identified, the gamma-delta variant of the disease is recognized as a distinct clinicopathologic entity in the WHO classification system.^{93,122,183} As TCR $\alpha\beta$ HSTCL has not been reported in the veterinary literature, it will not be discussed.

In both the human and veterinary literature, HSTCL is considered to be rare. In humans, approximately 200 cases of HSTCL have been reported, which represents less than 1% of all human lymphomas. The incidence of HSTCL in veterinary medicine is uncertain, but the small number of published reports since its initial description in 2003 suggests that it is similarly rare. HSTCL has been described in three reports, encompassing four dogs and one horse.^{27,65,161} Although a definitive diagnosis of HSTCL in the cat has yet to be published, a report of erythrophagocytic T cell lymphoma, which shares clinical and clinicopathologic features of HSTCL has been published.²⁵

HSTCL in humans may be associated with immunodeficiency, particularly in patients undergoing long-term immunosuppression associated with organ transplantation or inflammatory bowel disease.¹² Approximately one-third of human cases have been associated with organ transplantation and other conditions that require immune-modifying agents. The mechanism is unknown, but may be related to disruption of T cell negative regulation.

Human patients with HSTCL typically present with marked hepatomegaly, splenomegaly, or both, in the absence of lymphadenopathy.^{12,27,92,209} They also have cytopenias of more than one lineage.^{12,209,212} In animals with HSTCL, the clinical features include lethargy, fever, splenomegaly, anorexia, diarrhea, and pancytopenia.

The diagnosis of human HSTCL generally requires histological and immunohistologic evaluation of bone marrow and spleen, tissues in which the vascular tropism characteristic of the neoplastic cells can be identified.^{12,165,209} Infiltration by atypical cells within marrow sinusoids is common, but cellular identification is often difficult, because the infiltrating population is often subtle and requires immunohistochemistry. In humans, the neoplastic cells in HSTCL are most commonly CD2, CD3, CD38, CD56-positive cells with variable expression of CD7 and CD16.^{165,203} According to the limited veterinary patient phenotype data, the cells in four cases of canine HSTCL are described as CD3, CD11d, and TCR $\gamma\delta$ -positive.^{27,65} Additionally, activated hemophagocytic histiocytes may be admixed with the neoplastic T cells.¹² Despite its common involvement, evaluation of liver is not recommended to diagnose HSTCL in people because the histologic pattern is more

commonly consistent with an inflammatory rather than a neoplastic process.

Given the limited number of cases in animals, an algorithm to diagnose veterinary patients with this disease remains to be constructed. Despite this, features similar to human HSTCL (prominent sinusoidal tropism in liver, spleen and bone marrow, and neoplastic cell CD3-positivity) have been reported in cases of canine HSTCL. However, in contrast to the humans, the antemortem evaluation of bone marrow through aspiration cytology, was not helpful in the two dogs in which it was performed.^{27,65}

The prognosis of HSTCL is poor; most human patients die within 16 months of diagnosis irrespective of therapy, and three of the five animals with this disease (two dogs and the horse) survived less than 5 days after diagnosis.^{12,27,65,161,209}

Peripheral T Cell Lymphoma, Not Otherwise Specified

PTCL-NOS is a WHO category created to encompass those peripheral T cell malignancies that cannot be readily classified into another defined group based on morphology, phenotype, or molecular characteristics. As such, the diagnosis of PTCL-NOS encompasses a heterogeneous group that includes 40–50% of all human T cell lymphomas, representing some of the most aggressive cases of NHL that often present as advanced disease in middle-aged to older people with involvement of lymph nodes, skin, liver, spleen, and bone marrow or blood.^{2,74,101} This disease is commonly associated with paraneoplastic syndromes including hypercalcemia, skin rashes, vasculitis, eosinophilia, and hemophagocytosis.¹⁷

The morphology of PTCL-NOS cells can range from uniform small nondescript lymphoid cells with indistinct nucleoli, to large anaplastic cells, to combinations of the two, but there is no correlation between cytological atypia and biological behavior.¹⁰⁵ Often, there are prominent reactive infiltrates admixed with the tumor cells, and not surprisingly, the latter have variable phenotypes, with expression of CD3 being the only consistent finding.²¹¹ PTCL-NOS in people is most commonly a nodal disease of CD4 cells; however, extranodal tumors, generally of CD8 origin, also are seen.¹⁰⁵

The heterogeneous nature of this NHL subgroup is reinforced by their gene expression profiles, although these clearly distinguish PTCL-NOS from the two next most common human T cell lymphomas, AITL and ALCL.² Treatment for this disease is generally unrewarding, and intensification of chemotherapy does not seem to improve outcomes over treatment with standard CHOP-based protocols.⁵⁵

Given the paucity of immunolabeling reagents and the absence of large-scale studies describing precise subcategories of lymphoma in domestic animals, it would be tempting to include many T cell tumors in this category. However, dogs are the only species where some rigor has been applied to reach this diagnosis.^{60,63} PTCL-NOS appears to affect middle-aged to older dogs,

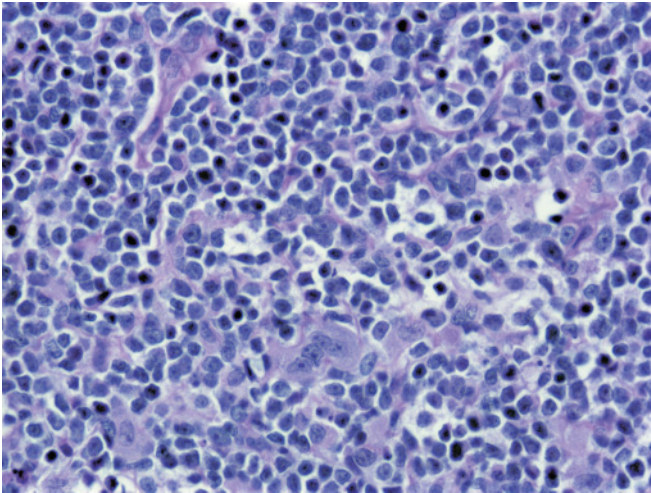


FIGURE 72.7 Peripheral T cell lymphoma, not otherwise specified in a lymph node from a dog. Notice the large, pleomorphic cells with prominent irregular nuclear contours and variable amounts of pale, foamy cytoplasm. Nucleoli are generally indistinct, although in this case they are apparent in some cells. Features that distinguish them from diffuse large B cell lymphomas (DLBCL) and LBTL, are that PTCL has only occasional macrophages (these are a prominent feature of DLBCL and very rare in LBTL) and it can have a prominent vascular network. H&E stain; original magnification $\times 500$.

causing regional or generalized lymphadenopathy. Morphologically, the tumor cells are pleomorphic, have prominent nuclear contour irregularity, indistinct nucleoli, and variable amounts of pale cytoplasm (Fig. 72.7). They mostly arise from CD4 T cells,⁶³ and in the authors' experience, they are frequently associated with paraneoplastic syndromes such as hypercalcemia. Like LBTL, these tumors are cytogenetically unstable, seem to harbor deletions of the CDKN2 locus, and respond poorly to multi-agent chemotherapy.⁶⁰

Natural Killer Cell Neoplasms

Natural killer cells are incompletely characterized in domestic animals other than the cow because there is a paucity of useful reagents that define their phenotype in humans and mice. Until now, a diagnosis of NK leukemia or lymphoma has been based on absence of T or B cell markers, and undetectable clonal rearrangements of lymphocyte antigen receptors. Yet, cells with morphologic characteristics and functions of NK cells can be isolated from canine blood,⁸² and the recent characterization of canine and feline CD56 may help to confirm the incidence of NK malignancies in these species.¹⁷⁹

Unclassifiable, High-grade Plasmacytoid Type Lymphoma

This subcategory of canine lymphoma was only recently described and is of particular interest given the disparity between its cytologic features and immunopheno-

type. The tumor cells resemble plasma cells; yet, they consistently express T cell markers. There is no category of human T cell lymphoma with comparable features, so this tumor may warrant classification in a separate category in dogs.⁶³

High-grade plasmacytoid T cell lymphoma appears to affect younger dogs and displays aggressive clinical behavior.¹⁵² Dogs have regional or generalized lymphadenopathy along with hepatosplenomegaly, mediastinal involvement, and bone marrow infiltration with infrequent atypical cells in the circulation.^{63,151} In one study, hypercalcemia was present in four out of nine cases and response to multi-agent chemotherapy was unrewarding, with a median disease-free survival of 3 months.¹⁵¹

High-grade plasmacytoid T cell lymphoma has a diffuse histological appearance with high mitotic index and frequent tingible body macrophages that create the typical starry sky appearance associated with diffuse B cell lymphomas.^{63,151} The malignant lymphocytes are of intermediate size, occasionally binucleate with variably clumped chromatin and abundant basophilic cytoplasm with perinuclear clearing, all morphologic features consistent with plasma cells. They express CD3 and CD8, but some cases may co-express CD4.^{63,151} The presence of nuclei with irregular, flower-shaped contours consistent with T cell origin in a tumor with plasmacytoid morphology is one feature that should encourage the pathologist to perform additional tests to reach a definitive diagnosis.

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Histiocytic Proliferative Diseases

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Overview

Histiocytic Differentiation and Canine Histiocytosis
 Canine Cutaneous Histiocytoma Complex
 Solitary Cutaneous Histiocytoma
 Immunophenotypic Studies
 Regression of Histiocytomas
 Metastatic Histiocytoma
 Langerhans Cell Histiocytosis
 Treatment of Histiocytoma Complex
 Canine Histiocytic Sarcoma Complex
 Localized and Disseminated HS
 Morphological Features of HS
 Immunophenotypic Studies
 Treatment of HS Complex

Canine Reactive Histiocytoses

Systemic Histiocytosis
 Cutaneous Histiocytosis
 Morphological Features of SH and CH
 Immunophenotypic Studies
 Pathogenesis
 Treatment Options in SH and CH

Feline Histiocytic Proliferations

Feline Progressive Histiocytosis
 Immunophenotypic studies
 Feline Pulmonary Langerhans Cell Histiocytosis
 Immunophenotypic studies
 Feline Histiocytic Sarcoma Complex
 Immunophenotypic studies

Acronyms and Abbreviations

APC, antigen-presenting cell; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (Lomustine); CH, cutaneous histiocytosis; CNS, central nervous system; DC, dendritic cell; EMH, extramedullary hemopoiesis; FPH, feline progressive histiocytosis; HHS, hemophagocytic histiocytic sarcoma; HS, histiocytic sarcoma; IHC, immunohistochemistry; LC, Langerhans cell; LCH, Langerhans cell histiocytosis; MF, mycosis fungoides; MH, malignant histiocytosis; NECL, non-epidermotropic cutaneous lymphoma; PCR, polymerase chain reaction; PLCH, feline pulmonary Langerhans cell histiocytosis; SH, systemic histiocytosis.

OVERVIEW

There are at least four well-defined histiocytic proliferative diseases that have been recognized in dogs. They are a frustrating group of diseases because it may be difficult to differentiate them from granulomatous, reactive, inflammatory, or lymphoproliferative diseases by examination of regular paraffin sections. The clinical presentation and behavior and responsiveness to therapy vary between the syndromes observed (Table 73.1). Clinical and pathological images of canine histiocytic diseases, and details of histiocytic lineages are available on a web site maintained by the authors (<http://www.histiocytosis.ucdavis.edu>).

Canine cutaneous histiocytoma usually occurs as a single lesion in young dogs and spontaneously regresses. Metastatic histiocytoma is a rare example of aggressive

behavior of this solitary tumor. *Langerhans cell histiocytosis* covers a spectrum of disease from multiple cutaneous lesions, which individually resemble solitary histiocytomas, to multiple cutaneous lesions with progressive systemic involvement. *Cutaneous histiocytosis* (CH) presents with single or multiple lesions, which tend to wax and wane, and may even spontaneously regress. Few cases respond to corticosteroids, the remainder persist and may require more aggressive immunosuppressive therapy. *Systemic histiocytosis* (SH) is a familial disease of Bernese mountain dogs and also occurs sporadically in other breeds. Systemic histiocytosis presents with prominent skin manifestations identical to those seen in CH, but mucous membranes (ocular and nasal) and a variety of other organ systems, including lymphoid organs, lung, and bone marrow may also be involved. Although the lesions may wax

TABLE 73.1 Canine Histiocytic Disease Classification

Disease Group	Disease	Cell of Origin	Disease Progression
Histiocytoma complex	Histiocytoma	Epidermal LC	Spontaneous regression
	Langerhans cell histiocytosis	Epidermal LC	Widespread cutaneous/systemic disease. Spread beyond skin poor prognostic indicator
Reactive histiocytosis	Cutaneous histiocytosis	Interstitial DC (dermal DC)	Immunoregulatory disorder – limited to skin and lymph node. Responds to immunosuppression
	Systemic histiocytosis	Interstitial DC	Immunoregulatory disorder – skin, lymph node, mucous membranes, internal organs. Responds to immunosuppression
Histiocytic sarcoma complex	Localized histiocytic sarcoma	Interstitial DC	Initial solitary site – rapid dissemination
	Disseminated histiocytic sarcoma	Interstitial DC	Progression of localized disease; multicentric origin (equivalent to malignant histiocytosis)
	Hemophagocytic histiocytic sarcoma	Macrophage – splenic red pulp and bone marrow	Originates in spleen (± bone marrow); rapid spread to liver and lung

and wane, SH is a progressive disease that often requires continuous immunosuppressive therapy. *Histiocytic sarcoma* (HS) and *malignant histiocytosis* (MH) occur with high incidence in Bernese mountain dogs, Rottweilers, Flat Coat Retrievers, Golden Retrievers and sporadically in many other breeds. Histiocytic sarcomas occur as localized lesions in spleen, lymph nodes, lung, bone marrow, skin and subcutis, brain, and periarticular tissue of large appendicular joints. Histiocytic sarcomas can also occur as multiple lesions in single organs (especially spleen). Localized HS lesions rapidly disseminate to involve multiple organs. Hence, disseminated HS is difficult to distinguish from MH, which is a multisystem, rapidly progressive disease in which there is simultaneous involvement of multiple organs such as spleen, lymph nodes, lung, bone marrow, skin and subcutis. Response of HS and MH to chemotherapy is at best brief.

HISTIOCYTIC DIFFERENTIATION AND CANINE HISTIOCYTOSIS

The development of canine specific monoclonal antibodies for many of the functionally important molecules of macrophages and dendritic antigen-presenting cells (DCs) has enabled the identification of cell lineages involved in canine histiocytic disorders.^{1,3,14,19} The majority of histiocytic disorders involve proliferation of various DC lineages.

Histiocytes differentiate from CD34+ committed stem cell precursors into macrophages and several DC lineages, which include epitheliotropic DCs or Langerhans cells (LCs), interstitial DCs in many organs (e.g. dermal DCs in skin), and interdigitating DCs of T cell domains in peripheral lymphoid organs (see Chapter 8). Dendritic cells are the most potent antigen-presenting cells (APCs) for induction of immune responses in naïve T cells. Canine DCs have been best defined in canine skin. They occur in two major locations: within the epidermis LCs, and within the dermis,

especially adjacent to post-capillary venules (i.e. interstitial DCs or dermal DCs). Canine DCs abundantly express CD1 molecules^{12,14,19} which together with MHC class I and MHC class II molecules, are responsible for presentation of peptides, lipids and glycolipids to T cells. Hence, DCs are best defined by their abundant expression of molecules essential to their function as APCs. Of these, the family of CD1 proteins is largely restricted in expression to dendritic APCs in skin,¹² while MHC classes I and II are more broadly expressed.

The beta-2 integrins (CD11/CD18) comprise the major family of adhesion molecules on leukocytes, and as such are useful markers of leukocytic differentiation. CD11/CD18 expression is highly regulated in normal canine macrophages and DCs. CD11c is frequently expressed by DCs, while macrophages predominately express CD11b (or CD11d in the splenic red pulp and bone marrow).^{5,6} In diseased tissues, these beta-2 integrin expression patterns may be diversified.

Langerhans cells (intraepithelial DCs) and interstitial DCs are distinguishable by their differential expression of E-cadherin (LC+) and Thy-1 (CD90) (interstitial DC+). Lineage distinctions among histiocytes are best made via immunohistochemistry (IHC) performed on frozen sections (CD1, CD11b, CD11c, CD11d, CD18, CD90, MHC II, and E-cadherin expression). Less definitive, but useful distinctions can also be attained via IHC on formalin-fixed paraffin-embedded sections with panels of leukocytic markers developed for use in this format (CD3, CD11d, CD18, CD45, CD45RA, CD79a, and E-cadherin).

Successful interaction of dendritic APCs and T cells in response to antigenic challenge also involves the orderly appearance of costimulatory molecules (B7 family – CD80 and CD86) on dendritic APC, and their ligands (CD28 and CTLA-4) on T cells. Defective interaction of dendritic APC and T cells appears to contribute to the development of reactive cutaneous histiocytic proliferative diseases (cutaneous and systemic histiocytosis), which are related DC disorders arising out of disordered immune regulation (see below).

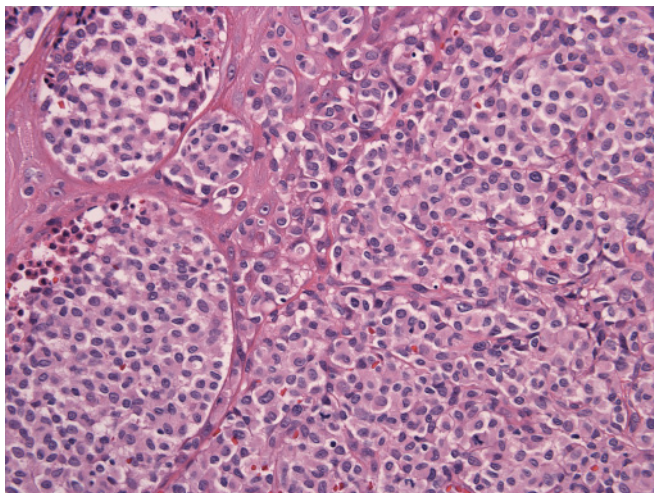


FIGURE 73.1 Canine cutaneous histiocytoma. Nest of histiocytes in the epidermis. Hematoxylin & Eosin stain.

CANINE CUTANEOUS HISTIOCYTOMA COMPLEX

Solitary Cutaneous Histiocytoma

Histiocytoma is a common, benign, cutaneous neoplasm of the dog. Histiocytomas usually occur as solitary lesions, which undergo spontaneous regression. The age-specific incidence rate for histiocytomas drops precipitously after 3 years, although histiocytomas do occur in dogs of all ages.²⁴ Recurrence of histiocytomas at the same or other sites is uncommon. The occurrence of multiple tumors is also uncommon. Epidermal invasion by cells of histiocytoma frequently occurs (incidence about 60%) and intra-epidermal nests of histiocytes resemble Pautrier's aggregates, characteristically found in epidermotropic cutaneous T cell lymphoma (mycosis fungoides or MF; Fig. 73.1). Epidermal invasion in histiocytoma, or presence of simultaneous multiple histiocytomas, especially in aged dogs, can present a diagnostic dilemma and distinction from MF and non-epidermotropic cutaneous lymphoma (NECL) is difficult without immunostaining for CD3 (T cell lymphoma) and CD18 (histiocytoma).¹⁷⁻¹⁹

Multiple histiocytomas are also readily confused with cutaneous histiocytosis on clinical appearance, although morphologically, histiocytomas are consistently epidermotropic and commonly epidermally invasive; these are not features of cutaneous histiocytosis, which is not focused on the epidermis. Instead the bulk of the lesion is formed by coalescence of perivascular infiltrates in the deep dermis and subcutis.

Immunophenotypic Studies

Immunohistochemistry is best performed on frozen sections of tumor or cytological preparations (not formalin-fixed material). Histiocytoma is readily distinguished from other histiocytic disorders and cutaneous lym-

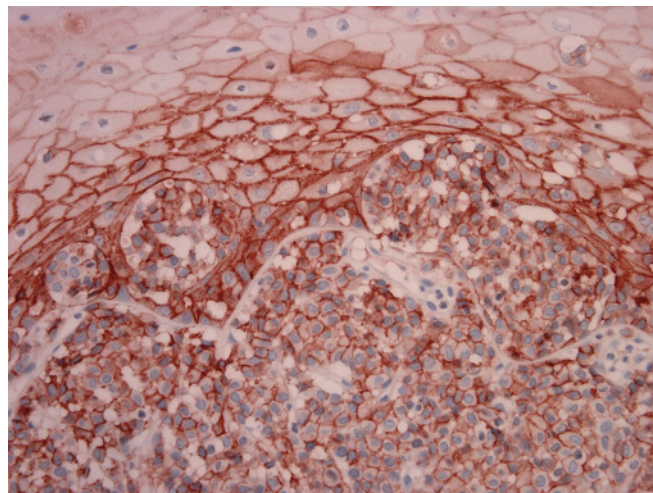


FIGURE 73.2 Canine cutaneous histiocytoma. E-cadherin expression by epitheliotropic histiocytes and by epidermal keratinocytes.

phoma with the aid of IHC. Histiocytomas have the phenotype of epidermal LC.¹⁹ They express CD1a, MHC class II, CD11c/CD18, and often E-cadherin. In formalin-fixed tissue, histiocytomas consistently express CD18 and commonly E-cadherin (Fig. 73.2). Among skin leukocytes, E-cadherin expression is unique to LCs. Langerhans cells utilize E-cadherin to localize in the epidermis via homotypic interaction with E-cadherin expressed by keratinocytes. E-cadherin expression has only rarely been observed in HS in canine skin and subcutis. Histiocytomas lack expression of CD4 and Thy-1, which are consistently expressed by histiocytes in CH and SH.

Regression of Histiocytomas

The factors that determine the onset of regression in canine histiocytomas are unknown. Evidence of regression is usually observed in lesions that have been present for only a few weeks, although regression can be delayed for many months. Regardless, regression is mediated by CD8+ $\alpha\beta$ T cells; only scant numbers of CD4+ T cells are observed in histiocytoma lesions. Migration of tumor histiocytes and/or tumor infiltrating reactive DC to draining lymph nodes could activate CD4+ T cells, which would assist in CD8+ cytotoxic T cell recruitment. Because massive CD8+ T cell infiltration is observed in all instances of histiocytoma regression, therapeutic intervention with the aim of immunosuppression should be avoided once a definitive diagnosis of histiocytoma has been reached, to allow unfettered cytotoxic T cell function.

Metastatic Histiocytoma

We have observed several dogs with solitary histiocytomas in which neoplastic histiocytes had migrated to draining lymph nodes resulting in complete obliteration

tion. In three of four instances regression of these lesions occurred spontaneously within 3–4 weeks.¹⁹

Langerhans Cell Histiocytosis

If histiocytomas occur in multiple cutaneous sites, there is a spectrum of clinical disease ranging from skin involvement only to skin, lymph node, and internal organ involvement. This spectrum of disease best fits under the umbrella of LCH.

Multiple histiocytomas, limited to skin or skin and draining lymph nodes, are best classified as cutaneous LCH. Multiple histiocytomas limited to skin appear to be more common in Shar Pei dogs, but can occur in any breed. Delayed regression of multiple histiocytomas can occur and lesions can persist for up to 10 months before onset of regression. In about 50% of instances, dogs with multiple histiocytomas are euthanized due to lack of regression of lesions and complications in management of the extensive ulcerated lesions that are frequently present. Multiple histiocytomas with lymph node metastasis have a poor prognosis, because spontaneous regression has not been encountered, and all affected dogs have been euthanized.

Multiple cutaneous histiocytomas with progression to lymph node and internal organ involvement are best classified as systemic LCH. Clinically, the lesions may be almost confluent in affected skin regions. Affected animals have all been euthanized and the time course is shorter than cutaneous LCH. There is one published account of a similar case without necropsy to verify internal lesions,²⁰ and the author has data on nine dogs with a similar presentation. Langerhans cell histiocytosis is also recognized as a rare disease of humans, in which marked variation in clinical behavior is recognized.^{7,22}

Treatment of Histiocytoma Complex

Solitary histiocytomas are either surgically removed or undergo spontaneous regression. Cutaneous LCH and systemic LCH are refractory to therapeutic intervention. Treatment with CCNU (Lomustine), which is commonly used to treat HS, has not been effective in LCH. Corticosteroids are contraindicated for reasons alluded to above. Spontaneous remission is possible in cutaneous LCH and this occurs in about half of the cases, unless lymph node involvement occurs. In one published report, response of cutaneous LCH to immunomodulatory therapy with griseofulvin was reported.²⁰ Others have tried immunomodulation with levamisole without success in cutaneous LCH.

CANINE HISTIOCYTIC SARCOMA COMPLEX

Localized and Disseminated HS

The HS complex encompasses a number of distinctive clinical entities. Histiocytic neoplasia, which originates at a single site, is called localized HS. This form of histiocytic sarcoma, which is often encountered on the

extremities, has the best prognosis if treated early by surgical excision or by amputation of a limb. When spread to sites beyond the local lymph node occurs, the disease is then termed disseminated HS; this is more likely to occur unnoticed when primary lesions occur in cryptic sites (e.g. spleen, lung, and bone marrow). This latter form of HS is most like MH. Malignant histiocytosis is an aggressive, histiocytic neoplasm, which arises in multiple sites simultaneously. Most lesions previously defined as MH are probably more correctly termed disseminated HS. The occurrence of true MH is difficult to establish because the lesions often occur in cryptic sites, and the existence of histiocytic neoplasia is only recognized after clinical signs have appeared and disease progression is advanced. HS and MH are capable of widespread metastasis; hence in time the two syndromes merge clinically and it is not always possible to differentiate true multicentric origin (MH) from widespread metastasis of disseminated HS. Also, it is never possible to know exactly how long the disease process has been operative. Hence, the perception is that both disseminated HS and MH follow a rapid clinical progression despite therapeutic intervention. This is certainly true once clinical signs are apparent, but the sub-clinical period is of unknown duration.

The HS complex of diseases is best recognized in the Bernese Mountain Dog, in which a familial association is apparent. Other breeds are predisposed to HS complex diseases and include Rottweilers, Golden Retrievers, and Flat-coated Retrievers.³ HS complex is not limited to these breeds and can occur sporadically in any breed. Primary lesions of HS occur in spleen, lymph node, lung, bone marrow, skin, and subcutis especially of extremities, and in periarticular tissues of the limbs. Secondary sites are widespread, but consistently include liver and lung when the spleen is the primary site, and hilar lymph node when lung is the primary site. Clinical signs include anorexia, weight loss, and lethargy. Other signs depend on the organs involved and are a consequence of destructive mass formation. Accordingly, pulmonary symptoms such as cough and dyspnea have been seen. Central nervous system (CNS) involvement (primary or secondary) can lead to seizures, incoordination, and paralysis. Lameness is often observed in periarticular HS. Regenerative anemia, thrombocytopenia, hypoalbuminemia and hypocholesterolemia have been consistently documented in hemophagocytic HS.¹⁶ Many of the cases have been mistaken for Evans' syndrome despite the lack of demonstrable IgG on the surface of red blood cells (RBCs).

Morphological Features of HS

Lesions of HS are typically destructive mass lesions with a uniform, smooth cut surface and are white/cream to tan in color. Lesions have a soft consistency and may contain discolored areas (typically yellow), which indicate area of necrosis. Lesions can be solitary or multiple within an organ (especially spleen). Periarticular HS has a distinctive appearance: it occurs

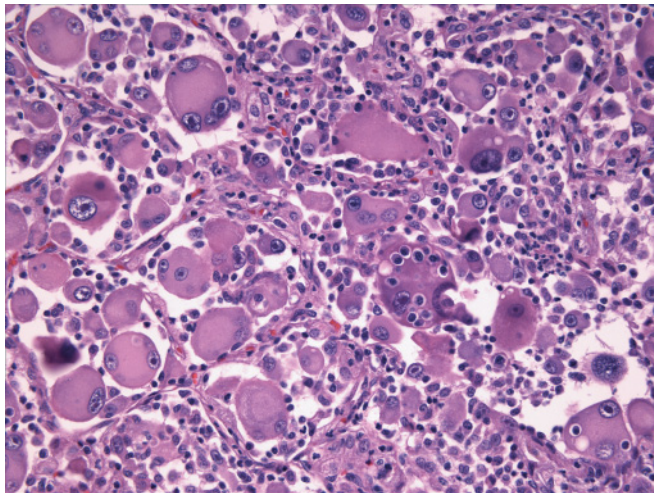


FIGURE 73.3 Histiocytic sarcoma. Cytologically atypical mononuclear and multinucleated giant cells in HS. Many cells are phagocytic. Hematoxylin & Eosin stain.

as multiple tan nodules located in the sub-synovium. These lesions may encircle the affected joint. Hemophagocytic HS does not initially form mass lesions in the primary sites (spleen and bone marrow). Typically, diffuse splenomegaly and ill-defined mass lesions are observed; the cut surface is dark red and the consistency is firm. The liver is usually bile stained (jaundice) and disruption of the lobular pattern due to metastasis is observed: marked liver involvement can occur in the absence of destructive liver masses.

The histological appearance of HS lesions is consistent regardless of location. Lesions are most frequently composed of sheets of large, pleomorphic, mononuclear cells and multi-nucleated giant cells, which show marked cytological atypia and numerous bizarre mitotic figures (Fig. 73.3). Some lesions may include spindle cell forms either alone, or mixed with the mononuclear cells and multinucleated giant cells. Pure spindle cell lesions resemble spindle cell sarcomas of diverse cell lineage. Confirmation of histiocytic lineage can only be achieved with IHC in these instances. Phagocytosis of RBCs, leukocytes and tumor cells occurs, but is not prevalent in most forms of HS. However, in hemophagocytic HS this behavior is amplified. Neoplastic histiocytes manifest marked erythrophagocytosis and the infiltrates obliterate the splenic red pulp and invade red pulp sinuses (Fig. 73.4). Foci of extramedullary hemopoiesis (EMH) occur within and adjacent to the tumor infiltrates in the splenic red pulp. Simultaneous involvement of bone marrow is frequent, and erythrophagia is observed here as well; these cases are probably equivalent to hemophagocytic MH in recognition of the simultaneous involvement of multiple sites. In some instances, the neoplastic infiltrates can be deceptively cytologically bland. The cytological appearance can be asynchronous between sites (e.g. spleen and bone marrow), which can contribute to diagnostic ambivalence if only one site is evaluated. Invasion of splenic red pulp sinuses portends invasion of the

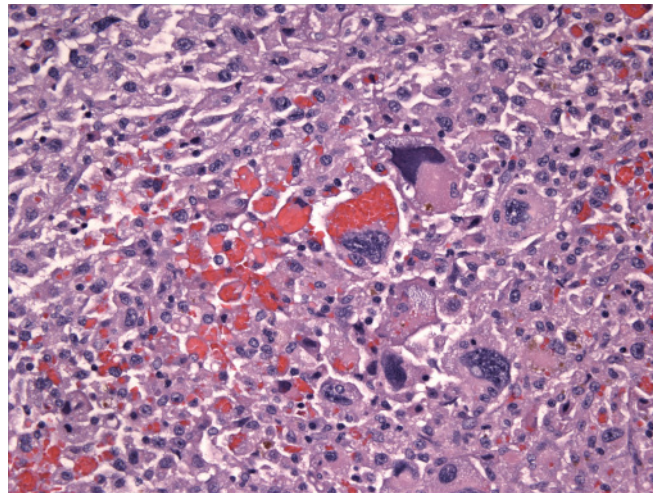


FIGURE 73.4 Hemophagocytic HS. Prominent erythrophagocytosis by cytologically atypical histiocytes in the splenic red pulp. Hematoxylin & Eosin stain.

hepatic sinusoids. In the early stages, liver metastases can easily be overlooked grossly and histologically, because histiocytic infiltrates creep along sinusoids and do not form discrete masses. Neoplastic histiocytes in hemophagocytic HS express a distinctive surface antigen profile much like that expressed by macrophages in splenic red pulp and bone marrow (see below).

In some instances, a secondary hemophagocytic syndrome can be confused with hemophagocytic HS. This has occurred most frequently with hepatosplenic T cell lymphoma, which is frequently accompanied by responsive anemia and thrombocytopenia. Demonstration of proliferative foci of CD3+ neoplastic T cells in spleen and liver in association with cytologically normal, hemophagocytic macrophages is necessary to resolve these cases.⁹

Immunophenotypic Studies

Histiocytic sarcoma lesions express leukocyte surface molecules characteristic of DC (CD1, CD11c/CD18 and MHC II) (Fig. 73.5). Diffuse expression of E-cadherin, Thy-1, and CD4 has not been observed in HS or MH in any site; this together with cytomorphology assists in the distinction of MH and HS from histiocytoma and reactive histiocytosis (SH and CH).^{1,3} In histiocytoma, the phenotype is quite similar to that of HS except for the expression of E-cadherin, which occurs in histiocytoma especially in the cellular infiltrate immediately adjacent to the epidermis. However, E-cadherin expression is often visibly weaker in tumor cells in the deep dermis. In reactive histiocytosis, the activated interstitial (dermal) DCs (CD1+, CD11c+, MHC II+, E-cadherin-), consistently express CD4, and Thy-1 expression occurs.

In hemophagocytic HS, histiocytes express CD11d instead of CD11c, and MHC II (Fig. 73.6). Expression of CD1 molecules is uniformly low, or occasionally moderate but with a patchy distribution. This phenotype is

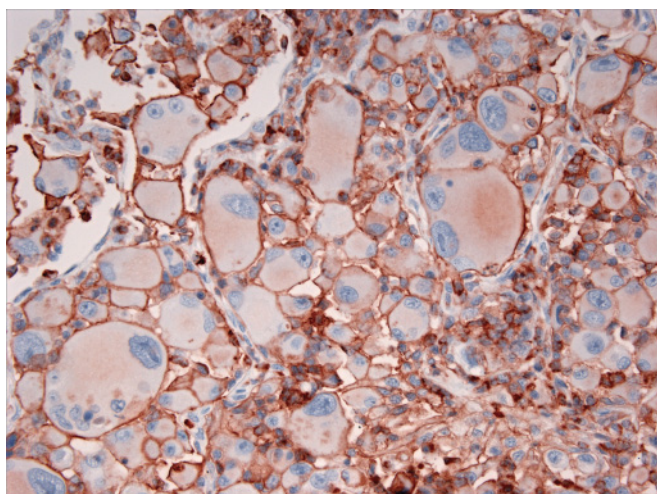


FIGURE 73.5 Histiocytic sarcoma. CD18 expression by atypical histiocytes. This most likely represents expression of CD11c/CD18.

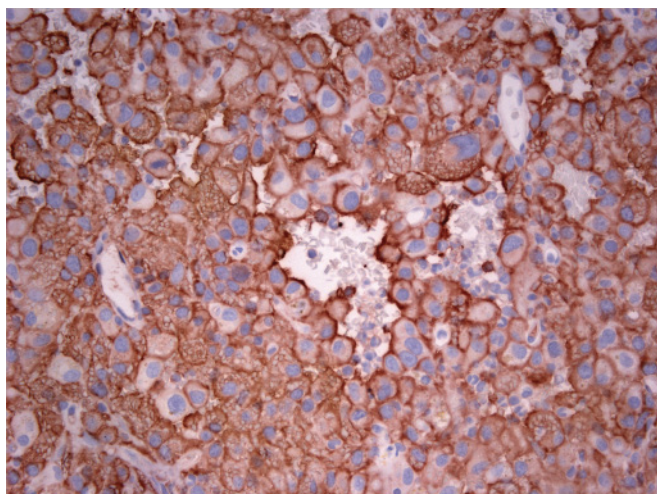


FIGURE 73.6 Hemophagocytic HS. CD11d expression by atypical histiocytes in the splenic red pulp – reflects expression of CD11d/CD18.

consistent with macrophage differentiation rather than DC differentiation, in which abundant expression of CD1 and CD11c is expected.¹⁶

The exact sublineages of DC involved in HS have not been determined in most instances. The most likely candidates include interdigitating DC in lymphoid tissues and perivascular interstitial DC in other involved tissues. Immunophenotyping and careful morphological assessment should also avoid confusion of HS and MH with the large cell form of T cell lymphoma (CD3+), and poorly differentiated mast cell tumors (CD18+ variable, CD45+, CD45RA+, Tryptase+, c-kit+).

Treatment of HS Complex

Localized HS affecting skin and subcutis has been cured by early surgical excision, which in some instances, has

been apparently successfully supplemented by local radiation therapy. In the case of periarticular HS, which occurs in the sub-synovial tissues of the extremities, amputation of the affected limb is necessitated by the inoperable nature of the primary lesions. It is important to establish that evidence of metastasis is absent via thoracic radiographs, abdominal ultrasound, and draining lymph node aspiration cytology before embarking on limb amputation. Disseminated HS (including MH) is not readily treated surgically, because even in the splenic form, early metastasis to the liver has often occurred. Chemotherapy with CCNU has been reported; the success depended on disease load. A small number of dogs with minimal residual disease had prolonged survival (>431 days); however, the median survival for all dogs was only 106 days. The authors identified hypoalbuminemia and thrombocytopenia as negative prognostic variables; dogs with these features survived less than 1 month.²³ The latter group of dogs may have included dogs with hemophagocytic HS.¹⁶

CANINE REACTIVE HISTIOCYTOSIS

Systemic Histiocytosis

Systemic histiocytosis was originally recognized in related Bernese mountain dogs.¹⁵ Systemic histiocytosis is a generalized histiocytic proliferative disease with a marked tendency to involve skin, ocular and nasal mucosae, and peripheral lymph nodes. The disease predominately affects young to middle aged dogs (2–8 years old). Systemic histiocytosis has been observed less frequently in other breeds (Irish wolfhounds, Basset hounds and others). Clinical signs vary with the severity and extent of disease and include anorexia, marked weight loss, stertorous respiration, and conjunctivitis with marked chemosis. Multiple cutaneous nodules may be distributed over the entire body, but are especially prevalent in the scrotum, nasal apex, nasal planum, and eyelids. Ulceration of the skin overlying the nodules is common. Peripheral lymph nodes are often palpably enlarged. The disease course may be punctuated by remissions and relapses, which may occur spontaneously especially early in the disease course. In severe disease, lesions become persistent and do not respond to immunosuppressive doses of corticosteroids.

Cutaneous Histiocytosis

Cutaneous histiocytosis is a histiocytic proliferative disorder that primarily involves skin and subcutis and does not extend beyond the local draining lymph nodes.¹³ Cutaneous histiocytosis occurs in a number of breeds. Evidence of spread beyond the skin would invoke the diagnosis of SH, a closely related disorder. Lymphadenopathy has not been emphasized in published reports, and has only been documented in a small number of our cases. The lesions occur as multiple cutaneous and subcutaneous nodules up to 4 cm in

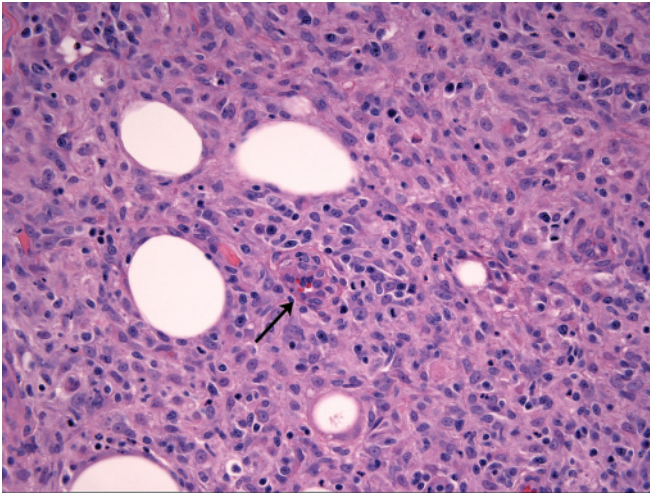


FIGURE 73.7 Cutaneous histiocytosis. Cohesive infiltrates of well differentiated histiocytes and fewer lymphocytes in the subcutis. Lymphocytes and histiocytes infiltrate a vessel wall (arrow). Hematoxylin & Eosin stain.

diameter. Overlying skin ulceration is common. Lesions may disappear spontaneously, or regress and appear at new sites simultaneously. Topographically, lesions may be found on the face, ears, nose, neck, trunk, extremities (including foot pads), perineum, and scrotum.

Morphological Features of SH and CH

The lesions of SH in most tissues consist of perivascular infiltrates of large histiocytes and variable populations of lymphocytes, neutrophils, and eosinophils (Fig. 73.7). The histiocytes frequently invade vessel walls and this may lead to vascular compromise and infarction of surrounding tissues, which contributes to ulceration of the cutaneous lesions. The widespread distribution of lesions of SH is only fully appreciated at necropsy. Histiocytic lesions have been observed in skin, lung, liver, bone marrow, spleen, peripheral and visceral lymph nodes, kidneys, testes, orbital tissues, nasal mucosa and other sites. In skin, the lesions of SH and CH are virtually identical. The lesions usually involve the deep dermis and subcutis. Involvement of the superficial dermis is inconsistent and epidermotropism of the histiocytes is not observed (Fig. 73.8). In CH the lesions are limited to the skin, but may involve the draining lymph nodes. It is important to note that the lesion topography of SH and CH are the inverse of histiocytoma, and this is an often overlooked feature in diagnostic decision-making.

Immunophenotypic Studies

Histiocytes in SH and CH express markers expected of DCs, including CD1, CD11c, and MHC II. However, lack of E-cadherin expression, and expression of Thy-1 (expressed by dermal DCs) and CD4 (a marker of DC activation) are consistent with an activated interstitial type DC phenotype.¹ In skin, dermal DCs are mostly of

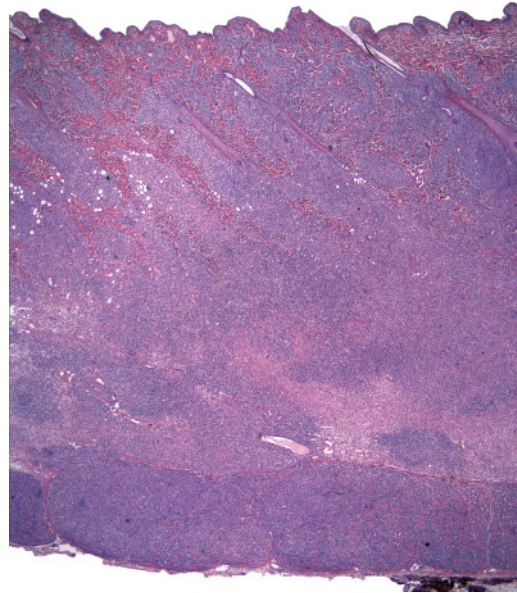


FIGURE 73.8 Cutaneous histiocytosis. Histiocytic infiltrates spare the superficial dermis and coalesce in the deep dermis and subcutis. Hematoxylin & Eosin stain.

interstitial DC type. In contrast, histiocytomas express an epidermal LC phenotype. Langerhans cells are intraepithelial DCs; they express CD1, CD11c, MHC II and E-cadherin. Langerhans cells lack expression of Thy-1, and do not express CD4 in the non-activated state.

Pathogenesis

The clinical behavior and consistent clinical response to immunosuppressive therapy with agents capable of profoundly inhibiting T cell activation has reinforced the concept that SH and CH occur in the context of disordered immune regulation. This may arise from defective interaction of DC and T cells in the resolution phase of an immune response. The end result of this dysregulated immune interaction is chronic proliferation of DC and T cells. The initiation of the process is probably antigen-driven, although studies to identify the nature of antigens involved have not been exhaustively conducted. Hence, it is important to perform tests to rule out infectious agents in the workup of a reactive histiocytosis case (culture and/or special stains for microorganisms in tissue). The lesions can wax and wane over time and spontaneous regression without therapy has been observed. The lymphoid component of the lesion consists of predominately CD8+ $\alpha\beta$ T cells, whose numbers can vary markedly between lesions. These T cells can comprise up to 50% of the cells in some instances. The role played by T cells is unknown. T cells may be involved in a key way in the exaggerated proliferation and activation of DC via T cell derived cytokines such as GM-CSF and TNF α , which are known to influence the proliferation and differentiation of DC.

The author believes that the continued distinction of SH and CH as separate entities is no longer justifiable.

It would be preferable to consider them within the spectrum of reactive histiocytoses of interstitial DC origin, in which clinical outcome is predictable more by the distant migratory potential of proliferating histiocytes beyond the skin. In this view, CH and SH would be regarded as skin limited and systemic interstitial DC proliferations, respectively. A wide range of clinical behavior is to be expected within each grouping, with SH usually exhibiting more aggressive disease. Cutaneous histiocytosis and SH should not be confused with malignant DC disorders (HS and MH), which can occur in the same topographical locations, and in littermates in genetically susceptible breeds. Cytological and immunophenotypic differences can distinguish these diseases in most instances. There has been very little direct evidence of progression of reactive histiocytosis to HS. On a cautionary note, inflamed T cell lymphoma of skin can look very much like reactive histiocytosis. In such cases, polymerase chain reaction (PCR)-based analysis of the T cell receptor gamma locus will identify clonal T cell expansion in the lesion with good sensitivity (about 80%).²⁵

Treatment Options in SH and CH

Systemic histiocytosis has proven to be a difficult and frustrating condition to treat. Consequently, many of the early cases were euthanized. Originally we treated dogs with thymosin (derived from bovine thymus) because of reports of its effectiveness in human LCH cases.²¹ Some dogs appeared to respond to thymosin, but not consistently. The original rationale for using thymosin was that SH was likely an immunoregulatory disorder and not cancer.¹⁵ Once modern immunosuppressive drugs became available, we abandoned the use of thymosin. In the majority of instances corticosteroid treatment is ineffective, although steroids are effective in 10% or more of cases in controlling lesions. Hence, steroids are worth trying given the expense of alternative treatments.

Intractable cases are best treated with immunosuppressive doses of cyclosporine A (Neoral, Novartis, East Hanover, NJ) or leflunomide (Arava, Aventis Pharmaceuticals, Bridgewater, NJ). These drugs are potent inhibitors of T cell activation, and their ability to abrogate clinical disease supports the hypothesis that SH and CH are disorders of immune regulation. Treatment with these drugs is expensive and may be needed for life in dogs with continuously active disease. It is preferable not to invoke such powerful immunosuppressive therapy until disease progression is evident or troublesome sites are involved, since in some cases of CH (and even SH) spontaneous regression of lesions or episodic disease activation can occur. Cost of treatment can be substantially reduced by co-administration of cyclosporine A and ketoconazole. It is imperative to measure 12 hour plasma trough levels of cyclosporine A (with twice daily dosing); this is especially important if ketoconazole is co-administered. The 12 hour plasma trough target for cyclosporine A is 500–600 ng/mL. Neoral is the preferred cyclosporine A drug, because it

is a microemulsion preconcentrate with superior gastrointestinal absorption compared to Sandimmune oral concentrate (Novartis), which consists of cyclosporine A in an olive oil base. Hence, Neoral can be used at a lower dose.¹⁰ Neoral is marketed as Atopica (Novartis) for treatment of dogs with atopic dermatitis; its main advantage over Neoral is the convenient capsule sizes optimized for veterinary use.

FELINE HISTIOCYTIC PROLIFERATIONS

True histiocytic proliferative diseases in cats have not been extensively documented in the veterinary literature. At least three different histiocytic proliferative diseases have been recognized in cats; these include progressive histiocytosis, pulmonary LCH, and HS/MH.

Feline Progressive Histiocytosis

Feline progressive histiocytosis (FPH) behaves as a low-grade histiocytic sarcoma, which originates in skin from resident DCs.² The initial clinical course is indolent and morphologically the histiocytes are cytologically bland. However, in time, the lesions become more troublesome and morphologically there is a higher frequency of cytological atypia more consistent with HS. The lesions have been considered an analogue of canine histiocytoma, although spontaneous regression is not observed, unlike canine histiocytoma. Canine histiocytoma is a LC proliferation, but the cell of origin in FPH has features in common with dermal DCs, which are interstitial DCs. The initial presentation of FPH may be a solitary skin nodule, although usually multiple papules, nodules or plaques develop, measuring up to 1.5 cm in diameter. The nodules are firm, non-pruritic and non-painful. The surface is often alopecic and may be ulcerated. The lesions are mostly located on the head, lower extremities or trunk. Occasionally, the lesions are limited to one extremity. Feline progressive histiocytosis is a disease of middle-aged to older cats, the age ranging from 7 to 17 years. Sex or breed predilection has not been seen. The lesions may wax and wane but spontaneous regression does not occur. In general, the nodules progress in size and may coalesce to large plaques. In addition, new lesions may develop. Some cats develop lesions in lymph nodes and internal organs including the lungs, kidneys, spleen, and liver. Additional clinical signs vary depending on the internal organ systems involved. Feline progressive histiocytosis has a poor long-term prognosis as no successful treatment has been recognized to date.

Lesions consist of diffuse dermal histiocytic infiltrates, which may extend into the subcutis. The overlying epidermis is either intact or ulcerated. Histiocytes have irregular, vesicular nuclei and finely dispersed chromatin. Cytological atypia is present in a minority of lesions early in the course. Biopsies of later lesions reveal a higher incidence of multinucleated tumor cells, and occasional intra-lymphatic tumor cell aggregates

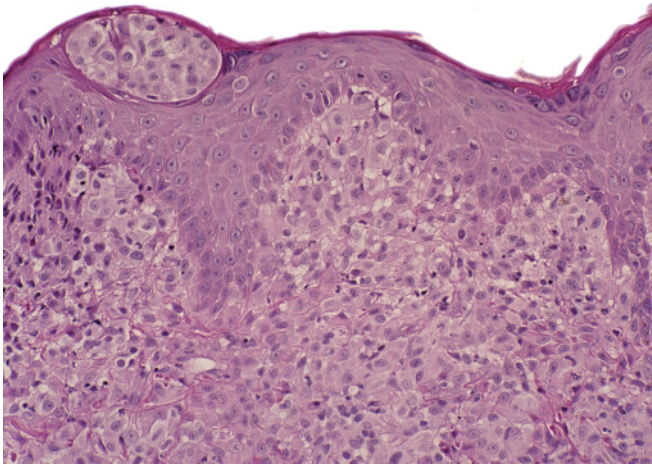


FIGURE 73.9 Feline progressive histiocytosis. Cohesive sheets of immature histiocytes infiltrate the superficial dermis and extend into the epidermis as a solitary focus. Hematoxylin & Eosin stain.

may be present. The mitotic activity varies and atypical mitoses are seen. Some cases are epitheliotropic, characterized by intra-epidermal single cells or cell aggregates; other cases lack epithelial involvement (Fig. 73.9). As lesions progress, cells may exhibit numerous cytoplasmic vacuoles and hence have a foamy appearance. The extent of reactive infiltrates, composed of dispersed lymphocytes and fewer neutrophils, varies between cases. Feline progressive histiocytosis has to be differentiated from xanthoma as well as multicentric round cell tumors, such as lymphoma and mast cell proliferation.

Immunophenotypic Studies

Histiocytes of FPH consistently express CD18, CD1, and MHC II. This immunophenotype is consistent with a DC origin.¹⁴ However, both the epitheliotropic and non-epitheliotropic lesions mostly lack expression of E-cadherin, and Birbeck's granules could not be found in the only case evaluated by electron microscopy. These features indicate that FPH is not composed of LCs, despite the existence of epitheliotropic infiltrates. The DCs are most likely of interstitial (dermal) type. The reactive lymphocytes are CD8+ cytotoxic T cells.

Feline Pulmonary Langerhans Cell Histiocytosis

Pulmonary Langerhans cell histiocytosis (PLCH) is a disease of aged cats (10–15 years old), which causes progressive respiratory failure leading to euthanasia.⁴ The cats have severe respiratory distress characterized by tachypnea and open mouth breathing. The symptoms can be acute or present for several months. Thoracic radiographs reveal a diffuse broncho-interstitial pattern of miliary to nodular opacities throughout all lung lobes. The original report described PLCH in three cats. Since then, the author has encountered two more cases. All have been diagnosed at necropsy. An

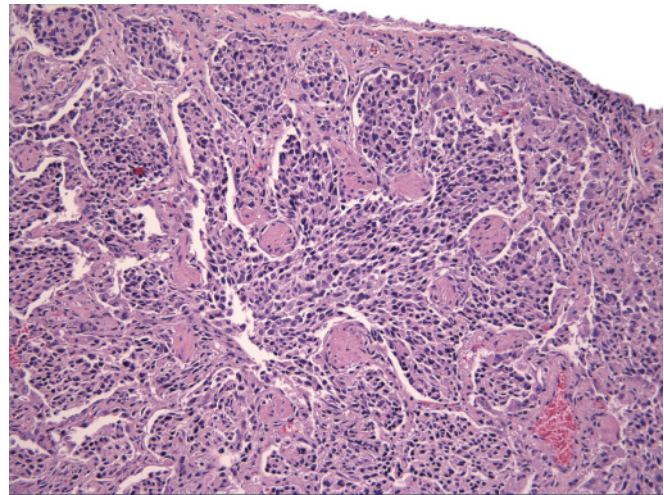


FIGURE 73.10 Feline pulmonary LCH. Cohesive sheets of histiocytes fill the alveolar duct and extend to the alveoli and pleura. Hematoxylin & Eosin stain.

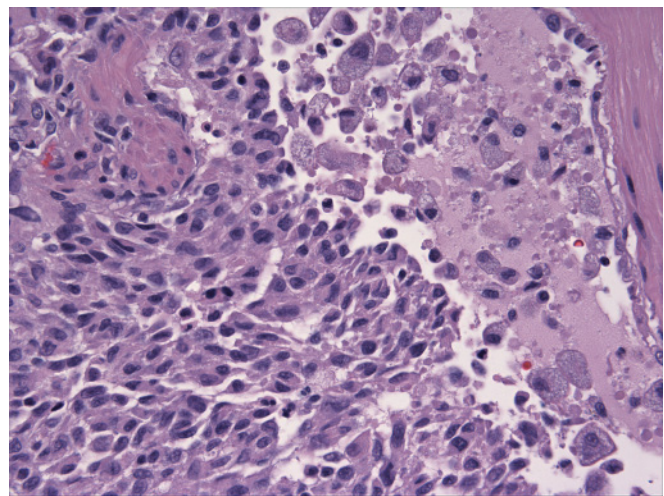


FIGURE 73.11 Feline pulmonary LCH. Moderately pleomorphic histiocytes with complex nuclear membranes invade the bronchial lumen, which is filled with foamy macrophages. Hematoxylin & Eosin stain.

infiltrative process involved all lung lobes, which were diffusely firm and entirely effaced by ill-defined, coalescing nodular masses (2–5 mm maximum dimension). Extra-pulmonary spread to pancreas and kidney was variably observed. Draining lymph nodes were also effaced.

Pulmonary lesions are characterized by histiocytic infiltrates within terminal and respiratory bronchioles. The infiltrates partially obliterate the airway walls and fill the lumens. Extension of the infiltrates into adjacent alveolar ducts and alveoli occurs (Fig. 73.10). Histiocytes form cohesive infiltrates with indistinct cell borders. They are moderately pleomorphic in cell size and nuclear morphology, which is often complex (Fig. 73.11). Transmission electron microscopy was used to

demonstrate characteristic Birbeck's granules in the cytoplasm of lesional histiocytes; these structures are only present in LCs and are formed in an endocytic process involving the C-type lectin, langerin.

Immunophenotypic Studies

The lesional histiocytes expressed CD18 and E-cadherin. E-cadherin is expressed by LCs, which use it to localize within epithelia.

Feline Histiocytic Sarcoma Complex

Localized HSs have been observed in cats. The poorly demarcated tumor masses were located in the subcutis of the ventral abdomen or extremities. Metastasis to draining lymph nodes occurs. These lesions may be the end stage of FPH, which would be indistinguishable from HS. Alternatively, primary HS may occur in the spleen.¹¹ Two of the cats in this study had lesions quite similar to those in canine HHS.¹⁶ Cats presented with anemia, thrombocytopenia, and hypoalbuminemia and splenomegaly like the canine cases, but studies of the cell of origin were limited due to lack of discriminating cell markers. We have seen similar cases in which erythrophagocytosis by bizarre histiocytes was the dominant feature, and the diagnosis was HHS. Investigation of cell lineage was conducted in a recent feline HHS case and a macrophage lineage was indicated based on lack of CD1 expression.⁸ Expression of CD11d by hemophagocytic histiocytes in feline HHS has not been evaluated; hence it is not known if feline HHS originates in splenic red pulp macrophages. Feline HS complex resembles the canine counterpart in terms of location of lesions and disease progression, but the incidence of feline HS is much lower. Feline HSs have a poor prognosis and most cats have been euthanized.

Feline HS shares morphological features with canine HS, including variable cytology encompassing mononuclear and multinucleated round cells, and discrete to aggregated spindle cells. Anisocytosis and anisokaryosis exist in tumor cells of all types. A moderate to high mitotic rate with bizarre mitotic figures is common. Prominent erythrophagocytosis is observed in feline HHS. Immunohistochemistry is required to differentiate histiocytic sarcomas from vaccine-induced sarcomas or anaplastic sarcomas with giant cells (incorrectly referred to as "malignant fibrous histiocytoma").

Immunophenotypic Studies

The round and spindle-shaped tumor cells in feline HS express CD18, CD1, and MHC II. This immunophenotype is consistent with a DC origin, although the precise

sublineage of DC has yet to be determined. The tumor cells lack expression of E-cadherin and hence, are not of LC origin. Feline HHS is reported to lack CD1 expression, which supports a macrophage origin.⁸

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Gene Therapy

BRUCE F. SMITH and R. CURTIS BIRD

General Approaches to Gene Therapy in Hematologic Neoplasia
 Vector Structures and Strategies
 Tumor Targeting
 Vector Detargeting
 Cell Specific Targeting
 Expression Targeting
 Tumor Cell Killing
 Suicide Gene Therapy
 Oncolytic Viruses
 Tumor Vaccination
 Immune Modulation
 Enhanced Antigen Presentation
 Enhanced T-Helper Activity

Modulation of Peripheral Tolerance and Regulatory T Cells
 Cytokine/Chemokine and Antibody Modulation
 Transplantation Therapy
 Non-specific Immune Stimulation
 Neoplastic Outcomes Resulting From Hematologic Gene Therapy
 Gene Therapy for Specific Veterinary Hematologic Neoplasms
 Leukemias and Lymphomas
 Osteosarcomas
 Future Directions in Veterinary Hematologic Gene Therapy
 Clinical Trials in Veterinary Species

Acronyms and Abbreviations

Ad5, Adenovirus serotype 5; APC, antigen presenting cell; CAV2, canine adenovirus 2; CRAd, conditionally replicative adenoviruses; CTL, cytotoxic T-lymphocyte; IACUC, Institutional Animal Care and Use Committee; IBC, Institutional Biosafety Committee; IFN, interferon; IL, interleukin; MHC, major histocompatibility complex; NK, natural killer cell; PEG-3, progression elevated gene-3; SCFV, single-chain Fv fragment; TAAs, tumor-associated antigens; TGF, transforming growth factor; TNF, tumor necrosis factor; Treg, regulatory T-lymphocyte.

GENERAL APPROACHES TO GENE THERAPY IN HEMATOLOGIC NEOPLASIA

Therapy for disease based on the introduction of novel genetic material has been discussed since the early 1980s. Initially, the concept of gene therapy was limited to replacement therapy in inherited metabolic diseases. However, that concept has been greatly expanded to include therapies for acquired diseases, including cancer. Now a maturing field, gene therapy has progressed into the clinics, with both human and animal patients enrolled in trials. Many innovative approaches have been identified including genetic vaccination, oncolytic replication competent viruses, and vector delivery of toxins or toxin converting genes. Much of the research that has been undertaken in the field of cancer gene therapy has focused on human cancers and a large amount of the preliminary evaluation of these strategies

has been performed in the laboratory mouse. These approaches have also been applied to a number of species of veterinary interest. The therapeutic application of genes in a variety of cancers in domestic animal species serves both to provide intermediate animal models in the evolution of these therapies for humans and to develop applicable gene therapies in these species themselves.

VECTOR STRUCTURES AND STRATEGIES

Gene therapy strategies are built around the delivery of genes to target tissues and cells. In most cases, this requires some further components in addition to the nucleic acids. These components are collectively known as vectors. Some common gene therapy vectors are shown in Table 74.1 and will not be discussed in detail here. Ideally, a vector should be able to survive the

TABLE 74.1 Gene Therapy Vectors

Vector	Insert Size (kb)	Target	Tropism	Comments
Retrovirus	~7	Depends on envelope used	Dividing cells	Integrates into host chromosome
Lentivirus	~7	CD4 (can be altered by using other env genes)	Quiescent and dividing cells	Integrates into host chromosome
Adenovirus	~7	CAR	Many cell types including liver, not tumor	Remains episomal, can be retargeted
"Gutted" Adenovirus	~30	CAR	Same as Adenovirus	Requires helper virus, can be retargeted
Adeno-associated virus	~4–5	Variable	Depends on serotype	Integrates in mouse, episomal in other species
Alpha herpes virus	~75	Unknown	Neurons	Highly pathogenic – requires significant attenuation for use
Plasmid (naked DNA)	~12	Variable	Depends on site of injection and any carrier molecules	Extremely stable long-term

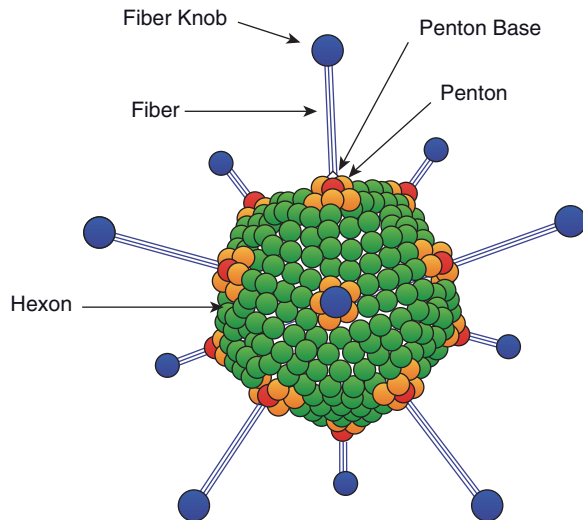


FIGURE 74.1 Structure of a typical adenovirus. The virus coat is an icosahedron consisting primarily of Hexon protein. At each vertex are five Penton and one Penton base proteins. The Penton base serves as the insertion site for the trimeric fiber protein, which forms a rod and a knob. Fiber knob is the binding site of CAR (coxsackie adenovirus receptor).

physiologic milieu, effect entry of the target cell across the plasma membrane and deliver its payload of nucleic acids to the nucleus, where gene expression occurs. A variety of different vectors have been identified, with most based on known viruses, which are essentially highly evolved gene delivery machines. These vector systems typically use engineered viruses, where critical genes for viral replication have been removed, rendering the virus replication incompetent (Fig. 74.1). In order to produce infectious virus particles, these genes must be provided *in trans*, by engineered packaging cell lines. As will be discussed in more detail below, subtle variations in these lines can result in innovative changes in vector targeting.

TUMOR TARGETING

The need to specifically target tumor cells within the body is obvious. Because of the nature of gene therapy, it presents unique opportunities to target tumor cells on a systemic basis, promoting tumor killing without affecting intervening cells. This can be particularly useful in treating widely disseminated metastases which might not be practical to approach either surgically, or with radiation. In gene therapy, targeting can be accomplished at several levels by combining targeting modalities, including vector detargeting, ligand based targeting and the use of expression targeting. Ideally, targeting of gene therapy for cancer patients consists of a combination of approaches, with each approach adding a layer of specificity and therefore safety and efficacy to the eventual therapy (Fig. 74.2).

Vector Detargeting

Many vectors have specific endogenous affinities that may or may not be appropriate for therapy. For example, Adenovirus serotype 5 (Ad5) may infect a wide variety of cell types through the CAR receptor; however, this receptor is often poorly expressed in many tumors. To assure cell-specific uptake of the vector, this receptor binding can be ablated to “detarget” non-tumor cells. There are several approaches to accomplish this. Antibodies to the cell-specific portion of the vector can be used to mask the binding site. For viral vectors, the viral site that binds to the target cell may either be modified to change binding or ablated to eliminate binding.

Cell Specific Targeting

There are multiple ways to achieve cell-specific targeting of gene therapy vectors including physical restriction of the vector, pseudotyping, genetic modification

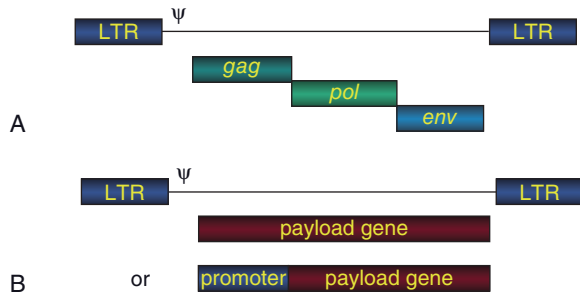


FIGURE 74.2 Genome structure and map of a typical mammalian retrovirus and a derived gene therapy vector. (A) A typical retrovirus encodes three major genes including *gag*, encoding the membrane glycoprotein, *pol*, encoding the reverse transcriptase (RNA-dependent DNA polymerase) and *env*, encoding the viral capsid protein. The retroviral genome also includes two long terminal repeats (LTRs) which encode the enhancer/promoter complex regulating viral gene expression and which are responsible for viral genome integration into the host genome. The bidirectional enhancer component of the LTR is also responsible for the insertional oncogenic activations associated with retroviral genome insertion events observed in gene therapy trials. (B) In gene therapy vectors derived from such viruses, the three major viral genes can be replaced with a gene therapy payload gene as long as the LTRs and the packaging sequence (ψ) are included and the viral particles are assembled in a packaging cell line that contains a helper retrovirus to provide the *gag*, *pol*, and *env* gene products. Expression of the payload gene may be driven off either the intrinsic LTR promoter (top) or an exogenous promoter can be inserted (bottom).

and redirection with antibodies to cell surface markers. Depending on their mode of action, vectors can be injected into specific and restricted body compartments that will limit their spread. These include direct injection into the tumor. In many tumors, direct injection requires multiple injections at numerous locations to treat the entire mass. A regional administration approach may also be used. Commonly, this method isolates the vasculature to a single limb and allows intravascular administration to the limb. Briefly increasing intravascular pressure in the isolated limb may allow vectors to freely cross the vascular endothelium and enter tissue beds. When perfusion is re-established, there may be some washout of vector into the general circulation.

Vectors may also be administered systemically and targeted to specific cells using cell surface markers. This approach requires that markers be identified that are restricted to a particular cell type, or a limited subset of other cells. In the case of tumor cells, these may be tumor-associated antigens (TAAs), which are typically cell surface proteins that are normally expressed during development. Given the variety in natural tissue tropism among virus families, it may be possible to exploit the tissue specificity of a particular virus, either through using that virus, or through pseudotyping, a process whereby one virus is given the “coat” proteins of another virus. For example, the tropism of human Ad5, which has an extensive range of target tissues that

express CAR, can be restricted to a different subset of cells if the Ad5 fiber is replaced by the Ad3 fiber protein.³⁰

Receptors that are not specifically viral receptors may be used to target cells through ligand-receptor interactions. These interactions are often highly specific, and will only target those cells expressing the receptor. This approach may also result in activation of the cellular signaling pathway that is normally the target of the receptor. Depending upon the receptor, cell type and pathway, this may be beneficial, or it may prove detrimental. If the target is not a receptor, or activation of a receptor is undesirable, antibodies may be used to target those cells. Conjugation strategies to attach the antibody to the vector can also add complications. It is also possible to identify peptides with targeting efficacy without any prior knowledge of the target cell’s protein inventory. Phage display libraries can be used to screen millions of potential peptides for their capacity to bind to specific cell types. These libraries can be screened in vitro with cultured cells or in vivo by injecting the library and then harvesting the appropriate tissues or cells. By screening for several rounds in the target tissue, it is possible to enrich for peptides or families of peptides with similar sequences and enhanced affinity for target tissue.

Targeting molecules, including peptides, ligands, and antibodies, can be used to target in several ways. Viral vectors can be engineered so that these moieties are expressed in an appropriate location that both detargets the vector and retargets it at the same time. Care must be taken though that the added targeting molecule does not alter or destroy the ability of the virus to assemble, bind, infect or replicate.

Non-genetic approaches to targeting vectors usually include “biphasic” adapters of some type. These adapters are often engineered fusion proteins that consist of two binding moieties, one to bind the vector and the other to bind the target cell. This approach has been demonstrated using Ad5 vectors. In this case, the adapter was created from an SCFV (single-chain Fv fragment) that binds to the Ad5 fiber knob region, blocking CAR binding and therefore detargeting the vector, and either a second SCFV, or a receptor ligand, such as CD40L. These adapters have the advantage of allowing the vector to be produced as a generic reagent using the common packaging lines or techniques, and then mixed at the bedside with the targeting adapter to allow customized targeting of the vector for each patient.

Expression Targeting

Targeting may also be achieved at the level of gene expression through the use of specific promoters. These may consist of tumor or tissue specific promoters such as the tyrosinase promoter in pigmented epithelium and melanoma. The ideal promoter for use in tumor cells is one that is normally active early in development and is therefore not active in normal adult cells. One example of this is the progression elevated gene-3

(PEG-3) which is active in many different tumor cells.²⁷ One disadvantage to these promoters is that they cannot be controlled. Conditional promoters are promoters that can be either induced or suppressed by the addition of another substance. Among the most frequently used are the Tet-on and Tet-off promoters, that are engineered bacterial promoters that have been adapted for use in eukaryotic cells. These promoters are regulated by the antibiotic tetracycline and can either deactivate or induce gene expression in the presence of the antibiotic. Such constructs permit the expression of the therapeutic gene in a temporally controlled fashion, either providing the needed protein at the correct time, or allowing protein synthesis to be disabled as a safety measure should toxicity occur. These promoters do not offer any tissue specificity.

TUMOR CELL KILLING

Ultimately, the goal of any genetic therapy is to kill cancer cells while sparing normal tissues. There are many different approaches to achieve this including the use of toxins, the induction of apoptosis, lysis of tumor cells with replication competent viruses and activation of an antitumor immune response.

Suicide Gene Therapy

Perhaps the most classical approach is known as “suicide gene therapy.” This method consists of transferring a prodrug converting enzyme gene to the tumor cells, followed by the administration of the prodrug. Expression of the gene results in the presence of the enzyme, which converts the prodrug to a toxic drug, in turn killing the target cell. An example of an enzyme/prodrug combination is herpes simplex virus thymidine kinase (HSV-TK) and the anti-herpetic “X”-cyclovir prodrugs (e.g. gancyclovir). HSV-TK metabolizes the prodrug into a nucleotide analog, that is then incorporated into the cell’s DNA during replication of the genome. The drug may also pass to neighboring cells through gap junctions resulting in a “bystander effect.” This results in killing greater numbers of cells than actually express the transferred enzyme. Killing is usually confined to tumor cells.²⁴

Oncolytic Viruses

Oncolytic virotherapy consists of the administration of replication competent viruses to tumor cells. These viruses are selected or engineered to specifically replicate in tumor cells, resulting in the eventual lysis of tumor cells. When the cell lyses, new viral particles are released to circulate and potentially infect additional tumor cells. The most commonly used oncolytic viruses are conditionally replicative adenoviruses (CRAd). In general, because of the species-specificity of viral replication, the CRAd must be derived from a virus that infects the target species. In humans, this is typically

Ad5. In dogs, canine adenovirus 2 (CAV2) has been adapted for use as a CRAd.¹³ Normally, this virus causes a mild respiratory disease in dogs, when inhaled, and is used as a vaccine for CAV1, which causes canine hepatitis. In this case, the E1 gene, an early gene product of the virus that is critical for replication, was placed under the control of the osteocalcin promoter to restrict replication to osteosarcoma cells. CRAds can be injected either intra-tumorally or intravenously. Intravenously injected CRAd has been shown to remain in the circulation longer than 48 hours, even in the face of pre-existing immunity.²⁵ Because the virus circulates, CRAds are considered to be an excellent method to target multiple distant metastases.

Tumor Vaccination

Tumor vaccines have been used with varying success for many years. These vaccines may consist of tumor cells or pieces, typically killed or fixed, that are injected back into the patient. In a refinement of this approach, specific tumor antigens, such as tyrosinase (or P100) in melanoma have been identified in a few tumors and have been used as antigens for tumor vaccines. The first DNA vaccine conditionally licensed for any species in the United States employs P100 as an antigen in an attempt to create an immune response to melanoma cells in the dog.¹ The type of immune response may also be critical to the success of this approach. A predominantly T-helper 2 (Th2) response with a high level of circulating IgG1 antibody, may not have any effect on tumor cells, while a predominantly T-helper 1 (Th1) response, with IgG2 and activation of cytotoxic T-lymphocytes (CTLs) may produce significant cell killing. For this reason, antigen presentation and adjuvants have critical effects on the ability of a tumor vaccine to raise a therapeutic response.

IMMUNE MODULATION

Several different approaches to immune modulation have been developed including enhanced antigen presentation strategies where tumor-specific antigens are introduced for presentation on antigen presenting cells or in which existing antigen recognition is enhanced to promote the breaking of immune tolerance. Systemic or local administration of antigen complexes, or nucleotides encoding them, has been attempted as have strategies whereby immune system components which suppress immunity are themselves suppressed. Gene therapy promoting the local expression of cytokines has also been adopted.

Gene therapy by introducing a foreign gene, such as green-fluorescent protein (GFP), has been used in a variety of species to promote immune targeting of neoplasias. This approach has been shown to promote immune responses to both the foreign gene target as well as tumor-associated antigens. Both cytotoxic T lymphocytes (CTLs) and antibody-based reactions have been promoted by this approach.

Enhanced Antigen Presentation

There is an abundance of data suggesting that tumors express specific antigens recognized by the immune system and in some instances these antigens induced immune responses to cause natural remission. A therapeutic strategy that could stimulate or enhance the effectiveness of immune recognition and induction of an effective immune response would be of immense value in the treatment of neoplastic disease, particularly in those cases of dissemination which are not surgically manageable. The basic strategy for forcing an immune response has involved different means of promoting or forcing tumor antigen presentation by antigen presenting cells (APCs). Such strategies have involved technologies such as protein antigen feeding to APC populations or transfection of DNA expression constructs or even tumor cell-derived mRNA population loading into APCs. Various APC populations, including allogeneic and autologous dendritic cells or even CD40 activated B cell populations have been antigen loaded.¹⁵ Another approach, designed to improve immune recognition, is based on creation of hybrid-cell vaccines through fusion of APCs with tumor cells.⁴ Hybrid-cell fusion constructs express both tumor-specific antigens and the necessary machinery needed for antigen presentation. Additionally, if they are MHC matched, T cell activation is also possible. Because dendritic cells are able to transport presented antigens to activate effector T cells in lymph nodes they have been suggested as promising candidates for the APC component of such hybrid-cell vaccines.^{4,5,10} Such experiments have been performed in normal healthy laboratory dogs providing proof-of-principle for successful development of tumor-specific immunity in this species.^{4,5}

Enhanced T-Helper Activity

Approaches to modify the T cell response to cancer have been attempted in dogs for a variety of cancers or to demonstrate proof-of-principle for strategies designed to enhance immune recognition of pan-tumor associated antigens associated with canine melanoma and mammary cancer.⁵ Application of such strategies to hematopoietic cancers is in a more formative stage; however, successful antigen loading of canine CD40-activated B cells has been demonstrated.²¹ Specific T cell responses were detected to model antigens and the intent is to apply this technology to spontaneous canine lymphoma.

Modulation of Peripheral Tolerance and Regulatory T Cells

The importance of regulatory T (Treg) cells in the recognition and development of a killing immune response to cancer has only recently been recognized, especially their contributions to suppression of the immune response to what would be otherwise considered self-antigens.² However, lack of appropriate antibodies to Treg antigens such as CD25 have inhibited investiga-

tions in species other than mouse and human until recently. A murine anti-FoxP3 antibody, which identifies this Treg-enriched transcription factor, has recently been shown to cross-react with canine FoxP3 putatively identifying canine Tregs.³ Such studies should provide the means to investigate the role of Tregs in canine intermediate models developed to investigate immunomodulatory strategies for treatment of cancer.

Cytokine/Chemokine and Antibody Modulation

The use of gene therapy to alter immune recognition through modification of cytokine, chemokine, or antibody expression has been an early approach attempted in a variety of cancer treatment strategies.¹⁹ Relevance has been demonstrated as canine lymphomas have been shown to express interleukin (IL)-2 receptors.¹² However, the toxicity and the prohibitive costs associated with systemic treatment with cytokines and other immunomodulatory molecules in intermediate and larger species has driven efforts to find technical solutions designed to force localized expression from inserted genes only at desired locations or tissues. Interferon (IFN)-gamma has been shown to modify major histocompatibility complex (MHC) and tumor-associated antigen expression and to induce antigen specific T cell responses. Interferon-gamma also appears to synergize with effects of interleukins, such as IL-6, and growth factors, such as transforming growth factor (TGF)-beta 1. There is also evidence that regression of canine Langerhans (dendritic) cell tumors are associated with expression of several cytokines including IL-2, tumor necrosis factor (TNF)-alpha and IFN-gamma.¹⁷ These investigations have suggested that such molecules have the potential to improve immune recognition, particularly if they are used in conjunction with other strategies designed to force presentation of tumor associated antigens.

There are also indications that IL-6 can antagonize TGF-beta 1-dependent inhibition of T cell activation. Canine tumor-infiltrative lymphocytes also synthesize IL-6 and may be recruitable to promote an antitumor activity.

Transplantation Therapy

There is a well developed history of immune modulation in dogs associated with a variety of transplant models as well as those approaches specifically designed to improve management of canine cancer. This has resulted in the development of a new transplantation approach to treatment of canine T cell lymphoma based on aggressive chemotherapy to induce remission followed by transplantation of hematopoietic cell lineages supported by subcutaneous injections of canine granulocyte colony-stimulating factor.^{11,20} This approach has also been demonstrated following radiation ablation.¹⁶ Although based on transplantation of related and at least partially DLA-matched hematopoietic populations, such approaches have been shown to promote disease-free survival of such patients for more than a

year. Additionally, the chimeric nature of the transplant was maintained in a stable form.

Non-Specific Immune Stimulation

Some effort has been applied to investigating the nature of nonspecific immune stimulation after the application of Toll-like receptor 9 ligands such as CpG containing oligonucleotides in primates, rodents, and dogs. Such nonspecific immunostimulants have a measurable effect on the innate immune system including natural killer (NK) cell activity. They also tend to promote Th1 type responses from the immune system and have been promoted for use as adjuvants.

NEOPLASTIC OUTCOMES RESULTING FROM HEMATOLOGIC GENE THERAPY

Gene therapy is a potent tool to alter the genomes of the target cell and organism, and it can be an extremely useful tool in the treatment of neoplasia. However, gene therapy has also been linked to the induction of tumors. In a clinical trial using retroviral vectors to treat X-linked severe combined immunodeficiency (X-SCID), at least four of ten boys developed leukemia. The therapeutic aspects were extremely successful, resulting in lifesaving immune reconstitution in nine of the ten patients. However, integration of the retrovirus in or near several proto-oncogenes, including LM02 in two patients, perhaps combined with expression of the transferred gene product, a cytokine, caused proliferation of those cells and subsequently, leukemia.³¹ A similar serious adverse event has been reported in a patient in an X-SCID gene therapy trial in England.¹⁴

GENE THERAPY FOR SPECIFIC VETERINARY HEMATOLOGIC NEOPLASIAS

The instances of successful treatment of veterinary hematologic neoplasias are few, but attempts have been made with varying degrees of success and notable problems with unanticipated side effects. Although many strategies have been investigated in cell culture *in vitro*, few have been translated to preclinical laboratory animal models or clinical cases. Among non-human and non-murine animal species, these neoplasias include leukemias of both the spontaneous and induced forms, and the more distantly associated osteosarcomas.

Leukemias and Lymphomas

Although few attempts to directly treat canine lymphoma using a gene therapy approach have been reported, there have been several investigations identifying gene targets that appear to play a causative role in development of canine non-Hodgkin's lymphoma and may be amenable to a gene therapy strategy based on genetic complementation *in trans*. The cyclin-

dependent kinase inhibitor p16/INK4A has been identified as a defective tumor suppressor gene in many of these canine lymphomas and could be amenable to repair by genetic complementation.^{9,23} Such an approach would seek to restore cyclin/CDK modulating activity to G1 phase of the cell cycle with the aim of providing the cell with the means to arrest or exit cell cycle via the p16/INK4A and retinoblastoma pathway. These cells appear also to harbor p53 defects making it likely that p53-dependent apoptosis is also defective.²⁶ Additionally, the IL-2 receptor has been identified as dysfunctional in canine lymphoma and may provide a surface marker for targeting or an additional target for gene therapy.⁸ Canine distemper virus is known to infect such lymphoma cells and has the potential to be used as a gene therapy vector if safety considerations can be addressed.²⁸ Although at a more formative stage than dogs, investigation of these tumors in cats has revealed some oncogene targets associated with feline lymphomas.¹⁸

Experimental treatment of leukemias has been attempted in dogs with mixed results owing to the nature of the side effects encountered. This strategy was based on a retroviral vector system and the introduction of a gene involved in imposing pattern formation on phenotype in developing animals. Homeobox (*HOX*) genes are a clustered family of genes involved in higher order developmental pattern regulation in multicellular animals. As such, these *HOX* genes play a critical role in the regulation of normal differentiation and appear to guide cells through an ever narrowing range of cellular fates or outcomes. The corollary for such an effect is thought to be a lack of control over phenotype should such genes become dysfunctional. Recent evidence has shown the potential of gamma-retroviral vectors in delivering *HOXB4* genes to hematopoietic stem cells and the expansion of repopulating cells.³³ The strategy was designed to replace neoplastic populations with genetically modified cells in which *HOXB4* cDNA constructs were inserted in an effort to impose such differentiated phenotypes on expanded populations of stem cells. This effect can be demonstrated in a variety of species and includes expanded proliferative potential and perhaps immortalization in some species, a trait only recently attributed to the *HOX* genes.³³ Control of aging appears to be highly homologous among mammals as the canine SIRT2 gene (a gene silencing regulator), an important regulator of lifespan in most multicellular species, has been identified and appears to be aberrant in function in spontaneous canine mammary tumor-derived cell lines.⁷

Transfer of *HOXB4* to hematopoietic stem cells using a gamma-retroviral vector in both dogs and macaques has revealed clear potential for leukemogenesis due to the presence of the genetic construct.³² All of the dogs transplanted (two survived transplantation) and half the non-human primates (1 of 2 macaques) developed leukemias related to the presence of the construct within 2 years of transplantation. There was clearly a difference among species as this effect had not been detected in prior mouse investigations and the potential for leukemia development was not predicted from these



FIGURE 74.3 Gene therapy clinical trial patient. A 10-year-old female spayed Rottweiler with osteosarcoma is shown receiving an oncolytic canine adenovirus intravenously following amputation of her affected forelimb. The high titer CRAd vector is administered slowly to reduce the potential of anaphylaxis.

experiments, although other experimental gene therapy trials in humans have uncovered such potential when using retroviral vectors. Innovative vector constructs have recently been reported for the investigation of ways to mitigate the predisposition for insertional mutagenesis of viral vectors in a mouse model.⁶ These vector constructs incorporated suicide genes to enhance drug sensitivity for transduced cells providing a means to eliminate leukemic cells.

Osteosarcomas

Osteosarcoma is among the most frequently occurring bone malignancies in dogs and has been promoted as a model application for the development of new conditionally replicative adenoviral gene therapy vectors.^{13,25} These adenoviral vectors have been targeted to deliver gene payloads to canine osteosarcoma cells in osteosarcoma patients. Although early in its development, pre-clinical trials in normal dogs have been conducted to determine dosage, safety, and levels of viral vector tolerance. To facilitate delivery but suppress uncontrolled spread, conditionally replication competent (CRAd) constructs have been developed. Measurable replication in several serial but diminishing peaks of the viral vector have been detected following treatment of tumor bearing canine patient animals (Dr. B.F. Smith, personal communication). Figure 74.3 shows the initial clinical patient to receive this vector.^{5,29} Trials using replication defective adenoviral vectors to deliver pro-apoptotic and immunomodulatory therapies also are in early phase clinical trials in dogs with osteosarcoma.²²

FUTURE DIRECTIONS IN VETERINARY HEMATOLOGIC GENE THERAPY

Gene therapy as a field is rapidly approaching its 25th anniversary. As such, the approaches being used are

maturing and gaining sophistication. Is gene therapy a magic bullet? Unquestionably, it is not. As with other therapeutic approaches to cancer, it will, in the end, be a combination of approaches, tailored to the specific tumor in the patient and designed to maximize tumor cell killing that will provide the most successful cancer management strategies.

Novel vectors are continually being introduced. In the next 5–10 years, vector development will focus on issues of enhancement of function. In particular, camouflaging vectors against immediate immune recognition and creation of vectors with novel tumor cell ligands are likely developments. In veterinary medicine, cost will drive the use of targeting molecules with the broadest possible application, especially if that is to treat multiple tumor types. To that end, cocktails of vectors with multiple and different targeting molecules may be developed to allow a single preparation to be used in all patients with a given tumor type. Targeting at the level of transcription may also become more broadly applicable with the use of promoters that are expressed in multiple tumors.

Vectors are also likely to undergo significant development in the coming years. Novel fusion proteins with enhanced specificities for prodrug conversion are currently being evaluated. In addition, payloads focused on inciting immune responses or activating cellular apoptotic pathways are gaining favor.

Clinical Trials in Veterinary Species

At the point that it becomes possible to conduct a “clinical trial,” that is an experimental administration of a novel therapeutic in client-owned animals, there is a critical need to interact with multiple constituencies. Depending on the organization, experiments in client-owned animals may be covered by the Institutional Animal Care and Use Committee (IACUC) which by law is required to review experiments with institutionally-owned animals, but which may expand its jurisdiction to client-owned animals. Alternatively or in addition to the IACUC, it may be necessary to satisfy a specific clinical trials committee, or other hospital-based quality assurance and patient safety regulatory bodies. Additionally, in the case of many biologics and certainly in the case of any vector using recombinant DNA, it is necessary to secure the approval of an Institutional Biosafety Committee (IBC). Once institutional approvals are in place, most trials proceed in a manner similar to human trials.

Initially, a Phase 1 trial is conducted, which is designed specifically to address safety. Some data may be generated in healthy animals in the preclinical phase; however, since these animals do not have tumors, it is necessary to evaluate safety in tumor bearing animals as well, as there can be important differences in physiologic response and immune status. In many cases, Phase 1 trials involve dose escalation to determine the maximum tolerable dose. If the Phase 1 trial is successful, then a Phase 2 trial may commence. Typically, this is a larger study designed to continue to gather safety

and dosing data, and to examine therapeutic efficacy. Some studies may combine Phase 1 and 2 trials to obtain efficacy data at an earlier time point. Following successful completion of the phase 2 trial, a larger, typically multi-center trial, known as a phase 3 trial, is undertaken to gather more definitive efficacy data, and to compare the experimental therapy to the current standard of care in a larger patient population.

In pet animals, the ability to perform clinical trials has been bolstered by the creation of the National Cancer Institute's (NCI) Comparative Oncology Trial Consortium. As of 2008, 16 veterinary schools were members of the consortium. When a party is interested in performing a large-scale clinical trial, they negotiate directly with NCI, which then coordinates the trial with the consortium members that would like to participate. This approach can either identify large numbers of cases for a major trial, or it can help accumulate rare cases where an investigator may not have sufficient caseload at their own institution.

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SECTION VI

Platelets

Mary K. Boudreaux

Platelet Structure

MARY K. BOUDREAU

Membrane Structure

- Microdomain Lipid Rafts
- Receptors

Cytoplasmic Structural and Membranous Elements

- Microtubules
- Open Canalicular System (OCS)
- Dense Tubular System (DTS)
- Mitochondria

Cytoskeleton

Granules

- Dense Granules
- Alpha Granules
- Lysosomal Granules

Acronyms and Abbreviations

Arp, actin-related protein; DTS, dense tubular system; GMP140, granule membrane protein 140; GP, glycoprotein; LAMP, lysosomal-associated membrane protein; LIMP, lysosomal integral membrane protein; OCS, open canalicular system; PADGEM, platelet-activated derived granule external membrane protein; PMCA, plasma membrane calcium ATPase; PtdIns, phosphatidylinositol; SERCA, sarco/endoplasmic reticulum calcium ATPase; VWF, von Willebrand factor; WASp, Wiskott-Aldrich syndrome protein.

MEMBRANE STRUCTURE

Microdomain Lipid Rafts

Mammalian platelets are derived from the cytoplasm of megakaryocytes. Platelet membranes are similar to those of other cells and are characterized by phospholipids arranged in a bilayer forming a hydrophobic core. Interspersed within the fluid lipid matrix are densely compacted microdomain lipid rafts primarily composed of sphingolipid and cholesterol molecules, and fewer numbers of protein molecules. These microdomain lipid rafts are capable of lateral mobility and enhance signaling in initial phases of platelet activation as well as enhance cytoskeletal reorganization events critical for normal clot retraction.⁹

Receptors

Proteins and glycoproteins, serving as specialized receptors important in platelet responses are present both within and outside the lipid rafts. Glycoprotein (GP) Ib-IX-V and GPVI, receptors involved in von Willebrand factor (VWF) and collagen binding, respectively, are examples of proteins primarily located within lipid rafts. Glycoprotein complex IIb-IIIa (GPIIb-IIIa), also known as integrin $\alpha_{IIb}\beta_3$,³ is the most prevalent glycoprotein complex on the surface of platelets and is

located outside of lipid rafts.²⁵ Although GPIIb-IIIa is not located within lipid raft microdomains, the complex does up-regulate phosphatidylinositols, in particular phosphatidylinositol(4,5)bisphosphate (PtdIns(4,5)P₂), as part of the outside-in signaling cascade. This process may be facilitated by interaction with tetraspanins located within microdomains distinct from lipid rafts.³³ PtdIns(4,5)P₂ induces the recruitment of several actin-modulating proteins resulting in the interaction of the cytoskeleton with the microdomains. The interaction of the cytoskeleton with lipid raft microdomains is necessary for sustaining the forces necessary for mediating clot retraction.⁹ GPIIb-IIIa receptors are important for mediating platelet aggregation by binding fibrinogen, although these complexes can also bind VWF and assist with early stages of platelet adhesion to the subendothelium. The major receptors present on the surface of platelets are summarized in Table 75.1.

CYTOPLASMIC STRUCTURAL AND MEMBRANOUS ELEMENTS

Microtubules

Just beneath the platelet membrane is a circumferential band of microtubules.³⁵ Microtubules are hollow, cylindrical structures composed of protofilaments formed by

TABLE 75.1 Classification and Function of Major Platelet Membrane Receptors^a

Classification	Function	References
Integrins		
$\alpha_{IIb}\beta_3$ (GPIIb-IIIa;CD41/CD61)	Known as the fibrinogen receptor. 50,000–80,000 receptors per platelet. Primarily mediates platelet aggregation; platelet activation is required to induce a conformational change resulting in ligand binding. Ligands include several RGD containing proteins including fibrinogen, VWF, thrombospondin, fibronectin, and vitronectin. Glanzmann thrombasthenia (GT) is due to mutations involving genes encoding α_{IIb} or β_3	60
$\alpha_v\beta_3$ (CD51/CD61)	Known as the vitronectin receptor. 200–300 receptors per platelet. Platelet activation is required to induce a conformational change resulting in ligand binding. Ligands include RGD containing proteins such as vitronectin, fibrinogen, and osteopontin	4, 62
$\alpha_2\beta_1$ (GPIa-IIa;CD49b/CD29)	Known as VLA2 on lymphocytes. 2000–3000 receptors per platelet. Major collagen adhesion receptor; platelet activation required for collagen binding via this receptor	47
$\alpha_5\beta_1$ (CD49e/CD29)	Known as the fibronectin receptor. Functions to supplement platelet adhesion; platelet activation is not required for fibronectin binding	56
$\alpha_6\beta_1$ (CD49f/CD29)	Known as the laminin receptor. Functions to supplement platelet adhesion; platelet activation is not required for laminin binding	29, 74
Leucine-Rich Repeat GPIb-IX-V	Known as the VWF receptor; also binds Mac-1 and P-selectin. Functions in platelet adhesion under high shear. 60,000 receptors per platelet. Bernard-Soulier syndrome is due to mutations in genes encoding GPIb or GPIIX	2
Seven Transmembrane		
Protease activated receptors (PAR)	Function as thrombin receptors. Platelets have 3 PAR receptor types; types vary with species. Human and dog platelets primarily have functional PAR1 and PAR4 types. Mouse platelets have functional PAR3 and PAR4 types	10, 16
ADP receptors	P2Y1 and P2Y12 have been identified on platelets. P2Y1 is coupled to Gq and initiates calcium mobilization resulting in shape change and reversible platelet aggregation. P2Y12 is coupled to Gi resulting in inhibition of adenylate cyclase and lowering of cAMP levels. P2Y12 activation is associated with irreversible platelet aggregation	61
Thromboxane receptor (TXA ₂ /PGH ₂)	Platelets possess two types of receptors, TP α and TP β , which are coupled to G proteins; TP α is coupled to Gs while TP β is coupled to Gi. TXA ₂ and PGH ₂ interact with both receptor types. While genomic studies suggest both types of receptors are present on platelets from cattle, horses, and cats, there is no genomic evidence for TP β receptors on dog platelets	79, MK Boudreaux, unpublished observations
Prostacyclin receptor (PGI ₂)	PGI ₂ receptors are coupled to Gs. Prostacyclin binding results in activation of adenylate cyclase, enhanced cAMP production, and inhibition of platelet activation	75
Platelet activating factor (PAF) receptor	PAF mediates platelet shape change and aggregation. PAF receptors are coupled to Gi and Gq. PAF can also initiate signal-dependent translation pathways in human platelets	85
Adenosine receptor	3 receptor subtypes have been detected on platelets. Adenosine receptor activation results in cAMP elevation and inhibition of platelet aggregation	1, 31
Adrenergic receptor	alpha2A-adrenergic receptors are present on platelets which are coupled to Gi. Epinephrine binding results in reduction of cAMP and potentiation of platelet activation. May play a role in enhancing thrombus stability	66
Serotonin receptor	Coupled to G-proteins leading to calcium mobilization and enhancement of platelet activation. Two subtypes have been detected on human platelets	1
Immunoglobulin/ITAM/ITIM		
GPVI-FcR γ	Major collagen receptor. Composed of two immunoglobulin C2 loops which bind collagen. Is complexed with FcR γ which contains an ITAM which is tyrosine phosphorylated during platelet activation	34
Fc γ RIIA	Immune receptor expressed on human but not mouse platelets. Binds IgG. Plays a role in heparin-induced thrombocytopenia	13
Fc ϵ RI	IgE immune receptor. Activation induces release of serotonin and RANTES and thus may play a role in allergic inflammatory responses	36
ICAM-2	Present on the surface of activated and non-activated platelets. The only β_2 integrin ligand on platelets; may play a role in leukocyte-platelet interactions	24

TABLE 75.1 Continued

Classification	Function	References
PECAM-1 (CD31)	Composed of six C2 immunoglobulin domains, a transmembrane domain, and a short cytoplasmic tail containing two ITIM domains. Plays dual roles as an inhibitor and enhancer of platelet activation. Has an inhibitory effect on ITAM-based collagen responses and also promotes $\alpha_{IIb}\beta_3$ mediated outside-in signaling	46, 80
CD47	Thrombospondin receptor; also known as integrin-associated protein (IAP). TSP binding results in enhanced platelet activation via inhibition of nitric oxide/cGMP signaling	40
Tetraspanins		
CD9	Present on resting platelet plasma membrane in association with $\alpha_{IIb}\beta_3$. May be involved in recruiting phosphatidylinositol kinase activity, specifically PI4K, to integrin-based signaling complexes	39
CD82	Studies in lymphocytes suggest a role in linking signaling through raft microdomains with the actin cytoskeleton. Presence on platelets not clear	21
CD151 (PETA-3)	Present on resting platelet plasma membrane in association with $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$. May play a role in integrin-mediated signaling	29
TSSC6	Associated with $\alpha_{IIb}\beta_3$ in resting platelets. As with other tetraspanins are associated with distinct microdomains that associate with lipid rafts during platelet activation, thus facilitating signal transduction events, including outside-in integrin signaling	33
Others		
P2X1	ATP receptor that enhances calcium entry. Plays a role in reinforcing and amplifying platelet activation	61
CD36 (GPIV, GPIIIb)	Ligands include thrombospondin, modified phospholipids, and long-chain fatty acids. Contributes to thrombus formation by binding to phosphatidylserine exposed on endothelial cell-derived micro-particles resulting in enhanced platelet activation	32
C1q	May play a role in classical complement pathway activation on platelet surfaces, particularly under high-shear induced platelet activation situations	63
Serotonin reuptake	Receptor is distinct from serotonin activation receptor. Responsible for serotonin transport across the platelet plasma membrane. Selective serotonin reuptake inhibitors inhibit platelet reactivity	72
Glycosyl phosphatidylinositol (GPI) anchored proteins	Platelets contain at least 5 GPI anchored glycoproteins on their surface. These include CD55, CD59, CD109, and prion protein. Platelets contain >96% of prion protein in human blood. Prion proteins are primarily present within alpha granule membranes and become expressed with platelet activation. Prion proteins are associated with platelet-derived microparticles and exosomes following thrombin-induced platelet activation	68
Thrombopoietin receptor	A member of the tyrosine kinase receptor family. Important for regulating platelet production. Receptors are internalized when bound by TPO. TPO binding does not activate platelets but does enhance platelet activation and may contribute to the adverse prothrombotic events observed in unstable angina in people	53

^aITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory domains; PETA-3, platelet and endothelial cell tetraspan antigen; RANTES, regulated on activation, normal T expressed, and presumably secreted; PI4K, phosphatidylinositol 4 kinase; TPO, thrombopoietin.

alpha-beta tubulin dimers arranged in a helical head-to-tail fashion. Microtubules are responsible for the maintenance of the disc-shaped form of the circulating platelet.⁴³ Under normal physiologic conditions, β 1-tubulin is solely expressed as a component of microtubules within megakaryocytes and plays a role in the orderly fragmentation of platelets from the megakaryocyte cytoplasm.⁷⁰

Open Canalicular System (OCS)

Connected to the surface of the platelet and extending deep within its cytoplasm is an extremely tortuous

maze of open interconnecting channels called the surface connected canalicular system or open canalicular system (OCS).⁸¹ This system was thought to be a remnant of the demarcation membrane system of megakaryocytes but the absence of the OCS in some mammals⁸⁶ and the presence of the OCS in thrombocytes of birds⁷⁷ and fish^{18,52} has cast some doubt as to its origin. The OCS can act as a conduit for the uptake of particles and for the release of granule contents by activated platelets. Particles such as viruses or bacteria can be taken up by the OCS; this activity usually results in platelet activation. Alpha and lysosomal granules have been documented to fuse and evacuate their contents

into the OCS around engulfed bacteria or viruses, but communication with the outside is always maintained, unlike phagocytic vacuoles of leukocytes. Thus, platelets are not considered to be true phagocytes.⁸² The OCS is everted to some extent during platelet activation and can thus serve to increase the number of surface receptors available for ligand binding since many of the same receptors found on the platelet surface also line the OCS.²⁷ Ruminant and equine platelets do not possess an OCS³⁰ although their megakaryocytes do possess a demarcation membrane system.⁷⁸ Platelets from these species do not enhance their surface area during activation, do not have a mechanism for uptake of particles, and release their granule contents by fusion of granules directly to the outer membrane. Figures 75.1 and 75.2 are electron micrographs of platelets

obtained from several species for comparison of overall appearance.

Dense Tubular System (DTS)

Another specialized cytoplasmic organelle is the dense tubular system (DTS). The DTS is a remnant of the smooth endoplasmic reticulum of the megakaryocyte and is located near the microtubules. Unlike the OCS, it does not communicate with plasma or granule membranes. DTS membranes contain cyclooxygenase and thromboxane synthase and thus participate in prostaglandin synthesis. The DTS is also a site for calcium sequestration by the platelet; calcium movement is facilitated by ATPase pumps termed SERCAs (sarco/endoplasmic reticulum calcium ATPases) which are

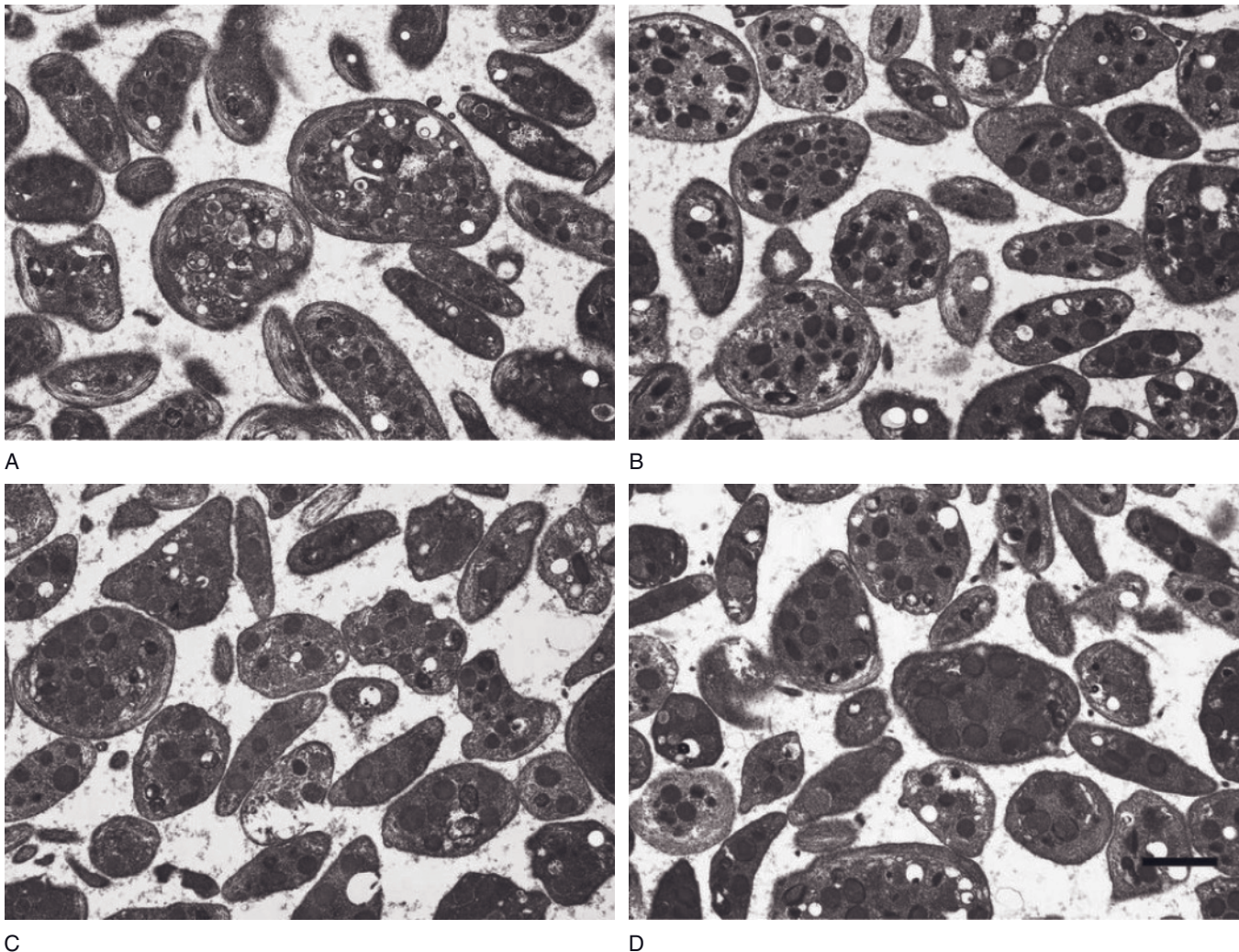
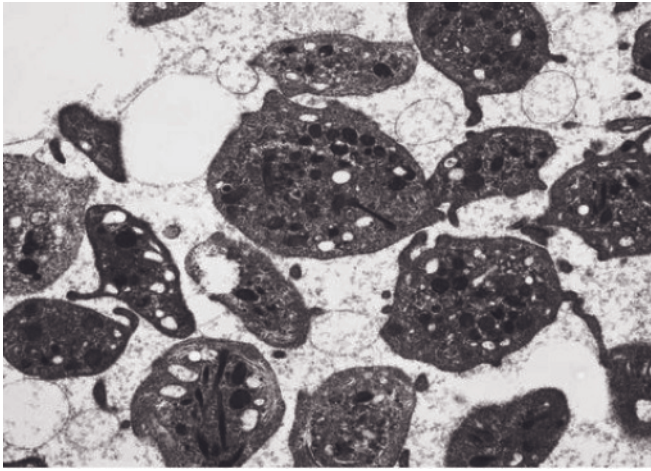
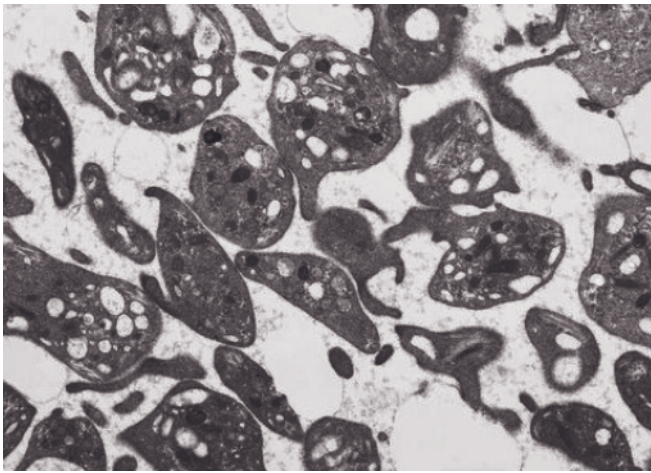


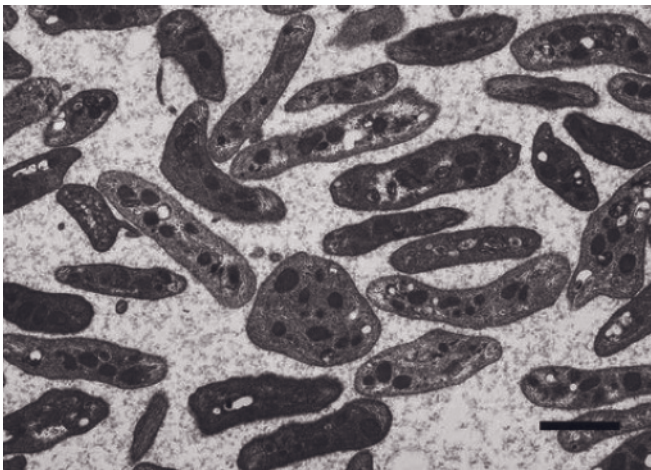
FIGURE 75.1 Electron micrographs of platelets obtained from alpaca (A), cow (B), goat (C), and sheep (D) for overall comparison of platelet morphology. Alpha granules appear light to dark gray and are prevalent and variably sized and shaped in these species. The electron dense content of most of the dense granules has been lost leaving vacuolar-like spaces. Microtubules can be observed in the periphery of some platelets. An open canalicular system is not present in these species. Bar = 2 μ m. (Courtesy of Dr. Maria Toivio-Kinnucan, Auburn University.)



A



B



C

FIGURE 75.2 Electron micrographs of platelets obtained from cat (A), dog (B), and horse (C). Alpha granules are numerous and variable in size and shape as seen with ruminant platelets. An open canalicular system is present in cat and dog platelets but absent in horse platelets. Bar = 2 μ m. (Courtesy of Dr. Maria Toivio-Kinnucan, Auburn University.)

tightly coupled to plasma membrane calcium ATPases (PMCA). Plasma membrane calcium ATPases function to move calcium ions to the extracellular medium while SERCAs pump calcium ions into the DTS. Calcium stores in addition to the DTS also exist in platelets but their specific location is poorly defined.⁵⁰

Mitochondria

Platelets have mitochondria important for maintaining energy and providing metabolic energy requirements during aggregation and secretion events. Mitochondrial membrane potential collapse is associated with platelet aging in circulation.⁶⁴

CYTOSKELETON

Platelets possess a cytoskeleton on the cytoplasmic side of the plasma membrane composed of actin, spectrin (fodrin), adducin, filamin, and glycoprotein Ib-IX-V (the VWF receptor). The membrane cytoskeleton interacts with an extensive cytoplasmic actin network. Adducin links the ends of cytoplasmic actin filaments to membrane associated spectrin strands. Filamin subunits bind to actin and to the cytoplasmic tails of the GPIb α subunits resulting in strengthening of the interaction of the spectrin/actin network with the plasma membrane.³⁵ During platelet activation adducin molecules dissociate from the ends of actin filaments allowing disassembly and reorganization of actin. Actin disassembly is promoted by gelsolin and cofilin. Gelsolin undergoes a conformational change in response to increased cytoplasmic calcium allowing the protein to interdigitate and disrupt actin filaments. Cofilin becomes activated as a result of dephosphorylation during platelet activation and functions to sever and promote actin disassembly along with gelsolin. Actin reassembly during the formation of filopodia and lamellipodia by activated platelets is mediated by the actin-related protein (Arp) 2/3 complex. Proteins involved in activation of the Arp 2/3 complex include cortactin and Wiskott-Aldrich syndrome (WASp) family proteins. Actin reassembly and myosin II binding and activation are critical for platelet spreading, granule movement, granule release, and clot retraction.

GRANULES

Platelets have three main types of storage granules, dense, alpha, and lysosomal, and they are heterogeneous in content and morphology.⁴⁹ Alpha and dense granules are thought to be derived from a common multivesicular body intermediate, and thus their membranes share several common components.⁸⁴ (Table 75.2).

Dense Granules

The dense granules or dense bodies, as their name implies, are electron dense when viewed using an elec-

TABLE 75.2 Granule membrane proteins

Classification	References
Alpha granules	
GTP-binding proteins	7, 48
Glycoproteins Ib-IX-V	8
Glycoproteins IIb-IIIa (CD41/CD61; $\alpha_{IIb}\beta_3$)	8
P-Selectin (CD62P)	41
Glucose transporter-3 (Glut-3)	37
PECAM-1	17
Vitronectin receptor	65
GPIV (CD36)	6
CD9	17
Osteonectin	11
Dense granules	
Hydrogen ion-pumping ATPase	20
GTP-binding proteins	54
Glycoprotein Ib	83
Glycoproteins IIb-IIIa	83
P-Selectin	41
Lysosome-associated membrane proteins (LAMP-2 and LAMP-3/CD63)	41, 42
Vesicular monoamine transporter (VMAT)	12
Nucleotide transporter (MRP4)	44
Orphan sugar nucleotide transporter (Slc34d3)	15
Lysosomal granules	
LAMP-1 CD107a	59
LAMP-2 CD107b	59
LAMP-3 CD63, LIMP	59
LIMP II LGP85	59

tron microscope, and serve as storage sites for adenine nucleotides, serotonin, calcium, and inorganic phosphates.^{57,67} The adenine nucleotide (ADP/ATP) ratio is roughly 1.5 within dense granules. The pool is metabolically inert and not available as an energy source. Serotonin is actively transported from plasma into dense granule cores and the serotonin concentration within dense granules is 1000 times higher than plasma concentrations. Dense granules contain 70% of the total calcium within platelets. This calcium pool is not mobilized during platelet activation, unlike the calcium pool within the DTS. Inorganic phosphates, calcium, serotonin, and adenine nucleotides are held together tightly within dense granules as a result of intermolecular forces, thus contributing to their stability and density.⁷³ Human and pig platelet dense granules contain high levels of lysolecithin,²³ however, lysolecithin was not detectable in the dense granules of rabbit platelets.¹⁹ Dense granules also contain ganglioside GM3 and phospholipids including phosphatidylethanolamine, phosphatidylinositol, phosphatidylcholine, and sphingosine.¹⁴ Organelle proteomics has led to the discovery of 40 proteins within dense granules, many of which had not been known to be present within this organelle. Dense granule protein categories include cell signaling proteins, molecular chaperones, cytoskeletal proteins, proteins involved in glycolysis, and proteins involved in platelet function.³⁸ Dense granule membranes contain

several receptors that become expressed on the plasma membrane as a result of dense granule fusion with the surface during the platelet release reaction. Some of these receptors are shared with other granule membranes and with the plasma membrane (Table 75.2).

Alpha Granules

The alpha granules, the largest and most numerous of the platelet granules, correspond to the azurophilic granules viewed by light microscopy. They are unique in terms of structure and packaging function. Structurally, alpha granules are composed of two major compartments, a dark centrally located nucleoid region and a peripherally located electron-lucent gray matrix region, that can be viewed at the electron microscopic level. The nucleoid region contains proteoglycans which confer stability to the granules. The nucleoid region is where beta-thromboglobulin and platelet factor 4 as well as other proteins are localized. Von Willebrand factor, multimerin, and factor V colocalize within tubular structures located in the outer part of the gray matrix region. Fibrinogen, thrombospondin, and fibronectin are primarily located in the gray matrix region in an area between the nucleoid and outer gray matrix region. Compartmentalization of VWF and fibrinogen within alpha granules has been documented in mouse, human, and porcine platelets.⁶⁹ Compartmentalization of proteins may serve to allow platelets to differentially release proteins in response to activation.⁷¹ Mouse alpha granules are more heterogeneous in terms of shape, size, and distribution compared to human alpha granules.⁶⁹ Alpha granules contain proteins that are synthesized by megakaryocytes as well as proteins that are endocytosed as platelets circulate in blood. Alpha granule proteins that are synthesized by megakaryocytes fall into two categories, those that are megakaryocyte-specific (such as beta-thromboglobulin and platelet factor 4) and those that are synthesized by other cells but are concentrated within platelet alpha granules (such as factor V and PDGF).⁷⁶ The latter proteins are sometimes referred to as platelet-selective proteins. The most well-recognized protein endocytosed by platelets is fibrinogen with uptake being mediated by the glycoprotein IIb-IIIa (integrin $\alpha_{IIb}\beta_3$) receptor. Proteins present in low levels within alpha granules such as albumin and immunoglobulin are passively taken up by platelets and do not require specific receptors. A proteomic analysis of human platelet alpha granule proteins has been recently published and provides a list of 284 proteins, 44 of which have not been previously identified within platelet alpha granules.⁵⁵ Alpha granule membrane proteins, much like dense granule membrane proteins, become expressed on the platelet surface after platelet secretion as a result of fusion of granules with the OCS or outer platelet membrane. The most well known alpha granule membrane protein expressed on the surface of activated platelets is P-selectin or CD62P (formerly known as granule membrane protein 140 (GMP140))⁴⁵ and as platelet-activated derived granule external membrane

protein (PADGEM)).⁵¹ Since its discovery, P-selectin has also been detected on the membranes of dense granules.⁴¹ Monoclonal antibodies have been developed to detect the presence of P-selectin on the surface of activated platelets in dogs.²⁶ Other proteins present within alpha granule membranes include GPIb-IX-V, GPIIb-IIIa, PECAM-1, vitronectin receptor, and GPIV (Table 75.2). Some of these membrane proteins have also been detected within the membranes of dense granules.⁸³

Lysosomal Granules

Lysosomal granules contain acid-dependent hydrolases including glycosidases, proteases, and lipases.²² Lysosomal granules can be identified in platelets and megakaryocytes using specific cytochemical stains.⁵ Secondary lysosomes have been detected in bovine megakaryocytes and platelets.⁵⁸ Lysosomal membranes also contain proteins that become exteriorized during the platelet release reaction (Table 75.2). Membrane proteins, which include lysosomal integral membrane protein (LIMP or CD63) and lysosomal-associated membrane proteins 1 and 2 (LAMP-1 and LAMP-2),²⁸ are heavily glycosylated to protect them from hydrolytic enzymes stored within these granules.⁵⁹ While initially LAMPs were thought to be solely present within lysosomal granule membranes, LAMP-2 and LAMP-3 have been detected within dense granule membranes as well.⁴² Lysosomal membrane proteins which mediate transport of ions and amino acids across the membrane necessary for maintenance of an acidic luminal pH are also present.

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Platelet Biochemistry, Signal Transduction, and Function

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Overview of Platelet Function
 Platelet Receptors and Activation Pathways
 ADP/ATP Receptors
 Collagen Receptors
 Thromboxane Receptors
 Thrombin Receptors

Platelet-Leukocyte Interactions
 Procoagulant Activity

Acronyms and Abbreviations

AA, arachidonic acid; CalDAG-GEFI, calcium diacylglycerol guanine nucleotide exchange factor I; CCL2, chemokine ligand-2; cPLA2, cytoplasmic phospholipase A2; CXCL8, chemokine IL-8; DAG, 1,2 diacylglycerol; DTS, dense tubular system; GIRK, G protein-gated inwardly rectifying potassium channel; GP, glycoprotein; ICAM-1, intercellular adhesion molecule-1; IP3, inositol triphosphate; ITAM, immunoreceptor tyrosine-based activation motif; LAT, linker for activation of T cells; LFA-1, lymphocyte function associate antigen-1; MLC, myosin light chain; NHE1, Na/H exchanger; PAK, p21-activated kinase; PAR, protease activated receptor; PE, phosphatidylethanolamine; PI3K, phosphoinositide-3-kinase; PIP, phosphatidylinositol; PIP3, phosphatidyl-inositol(3,4,5) trisphosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; PS, phosphatidylserine; PSGL-1, P-selectin glycoprotein ligand; ROCK, Rho associated coiled-coil-containing kinase; SLP-76, Src homology domain-containing leukocyte phosphoprotein of 76 kDa; TF, tissue factor; TP, thromboxane receptor; TRAP, thrombin receptor activation peptide; TXA, Thromboxane A2; VCAM-1, vascular cell adhesion molecule-1.

OVERVIEW OF PLATELET FUNCTION

Platelets are the first line of defense against bleeding at sites of vascular injury and are major contributors to thrombosis, inflammation and neoplasia. Platelets have cell surface receptors that recognize signals from their environment and communicate those signals to a complex network of biomolecules that include ions, proteins, nucleotides and phospholipids. While the details of many key networking pathways that elicit platelet responses are known, others remain to be elucidated. Platelet reactions orchestrated via these complex signaling networks include adhesion, aggregation, granule release, and procoagulant expression.

When molecules specifically recognized by platelets (agonists) bind to platelet receptors, they induce inside-out signaling resulting in structural changes in glycoproteins on the platelet membrane surface that then allow binding of proteins that mediate platelet adhesion and aggregation (Fig. 76.1; see also Chapter 81, Fig. 81.1). In turn, binding of adhesive proteins to receptors results in outside-in signaling events that promote and enhance platelet granule release, platelet aggregate and fibrin formation, and clot retraction.

This chapter reviews platelet signaling events communicated through the major activation receptors present on platelets as well as pathways and receptors involved in platelet-leukocyte interactions and expression of procoagulant activity.

Cross-talk between platelet receptors and signaling molecules is a key component of platelet activation, and much is still to be learned about the players involved in these events. Added to the complexity of understanding platelet signaling events is the recognition that platelets are capable of synthesizing proteins.⁶⁸ This capability, referred to as signal-dependent translation, will likely change current viewpoints of platelet involvement in many events including hemostasis, thrombosis, inflammation, and neoplasia.

PLATELET RECEPTORS AND ACTIVATION PATHWAYS

ADP/ATP Receptors

Platelets have three distinct P2 purinergic receptors, P2Y1, P2Y12, and P2X1.¹⁶ P2Y1 and P2Y12 are G

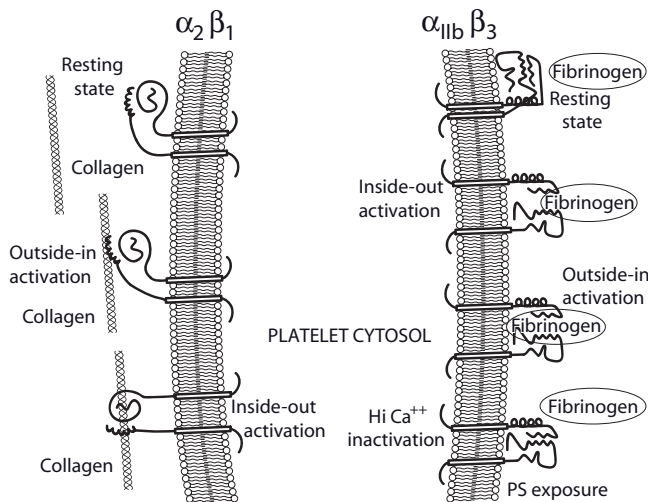


FIGURE 76.1 Platelet activation transforms the resting state of platelet membrane adhesion receptors for collagen ($\alpha_2\beta_1$) and fibrinogen ($\alpha_{IIb}\beta_3$) by altering their conformation states. Effector molecules generated by various signaling pathways in response to outside-in or inside-out signaling control the strength, reversibility or irreversibility of collagen or fibrinogen binding. Formation of tight and irreversible complexes consolidates platelet adhesion and aggregation. When platelet ionized Ca^{2+} is elevated for prolonged periods $\alpha_{IIb}\beta_3$ becomes secondarily inactivated and can no longer bind fibrinogen.

protein-coupled ADP receptors, mediating platelet shape change and aggregation responses, while P2X1 is a ligand-gated cation channel ATP receptor responsible for fast calcium entry and may play a role in collagen- and shear-induced platelet activation (Fig. 76.2).^{33,43,44}

P2Y1 is coupled to a $G_{\alpha q}$ protein subunit; signaling via $G_{\alpha q}$ results in activation of phospholipase C β_2 (PLC β_2), Rho A, Rac, and Src family kinases.²⁵ PLC β_2 mediates intracellular calcium mobilization and protein kinase C (PKC) activation via hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) within platelet membranes, to inositol triphosphate (IP3) and 1,2 diacylglycerol (DAG).^{30,44} IP3 mediates calcium mobilization from intraplatelet stores sequestered within the dense tubular system (DTS). DAG and ionized calcium activate diacylglycerol guanine nucleotide exchange factor I (CalDAG-GEFI) which in turn facilitates the displacement of GDP and loading of GTP within Rap1b. GTP-bound Rap 1b plays an important role in mediating the change in conformation of $\alpha_{IIb}\beta_3$ necessary for fibrinogen binding and platelet aggregation. The importance of CalDAG-GEFI downstream of PLC β_2 in mediating inside-out signaling events leading to activation of $\alpha_{IIb}\beta_3$ is illustrated in mice, dogs, and cattle with mutations in *CalDAG-GEFI*.^{5,6,14} All of these animals have a bleeding diathesis and impaired platelet responses to ADP and collagen.

P2Y1 activation results in platelet shape change as well as a reversible wave of platelet aggregation. Platelet shape change occurs as a result of both calcium-sensitive and calcium-insensitive pathways contributing to phosphorylation of myosin light chain (MLC).⁴⁵ The

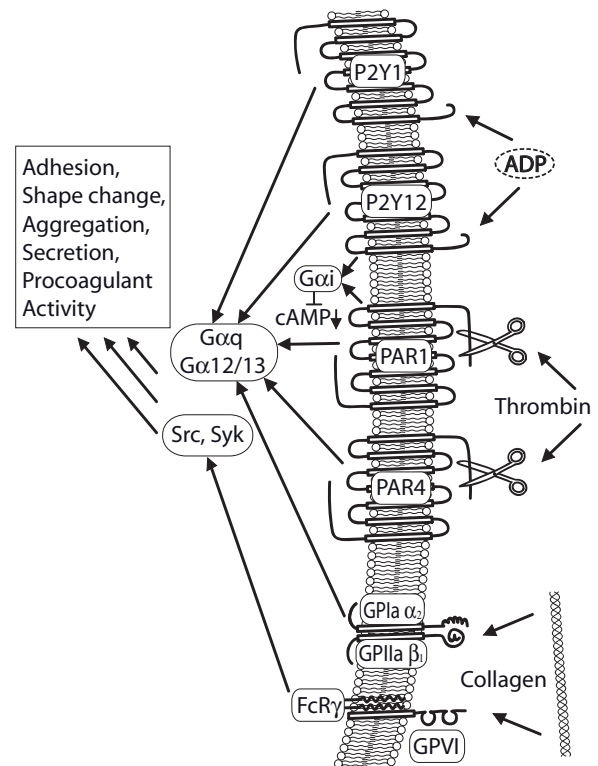


FIGURE 76.2 G protein-coupled receptors (GPCRs) link platelet-signaling pathways. The extracellular domains of paired receptors for ADP (P2Y1 and P2Y12), thrombin (PAR1 and PAR4) and collagen ($\alpha_2\beta_1$ and GPVI) interact with their specific ligands to mediate outside-in signaling pathways. The thrombin receptor is activated when thrombin binds to and cleaves the extracellular N-terminal domain unmasking a new tethered N terminus that binds to the second extracellular loop of the receptor. In contrast to $\alpha_2\beta_1$ which is a GPCR, GPVI signals collagen-initiated platelet activation through sequential activation of Src and Syk family tyrosine kinases.

calcium-sensitive pathway is mediated by calcium-calmodulin. The calcium-insensitive pathway is mediated by Rho A/p160ROCK downstream of $G_{\alpha q}$. Rho associated coiled-coil-containing kinase (p160ROCK) is a serine/threonine protein kinase that is directly targeted by Rho A. p160ROCK activation alters the balance between MLC phosphatases and MLC kinases in favor of MLC kinases, resulting in MLC kinase mediated phosphorylation of MLC necessary for platelet shape change.¹⁵ The RhoA/p160ROCK pathway is important in mediating the disruption of the microtubule ring necessary for transformation of platelets from discs to spheres and is also critical for maintaining the change in shape once shape change has occurred.⁴⁶ Rac, a Rho family GTPase that is activated by P2Y1, is thought to be important in mediating platelet spreading and anchoring via activation of a p21-activated kinase (PAK).⁴ Activated PAK is thought to activate the serine/threonine kinase LIM which targets and phosphorylates cofilin, inhibiting cofilin's ability to depolymerize actin.³

Rac activation by P2Y1 is potentiated by P2Y12 signaling.⁵⁸ In contrast, P2Y1 signals Src kinase

activation and the inhibition of the phosphoinositide-3-kinase (PI3K)-dependent phase of P2Y₁₂ signaling. This inhibition is thought to be an important negative feedback mechanism regulating platelet activation mediated by ADP.²⁵

P2Y₁₂ activation does not contribute to platelet shape change but is associated with platelet aggregation and granule release, thromboxane generation, expression of procoagulant activity and inhibition of adenylyl cyclase.⁵⁹ Engagement of the P2Y₁₂ is also required for amplification of platelet activation induced by ADP and low concentrations of other platelet agonists including TXA₂, collagen and thrombin. P2Y₁₂ is coupled to the heterotrimeric G protein complex G α i2 β γ .⁶⁶ ADP binding to P2Y₁₂ results in uncoupling of G α i2 and G β γ which act as separate signaling molecules. G α i2 inhibits adenylyl cyclase resulting in lowering of cAMP levels. G β γ dimers activate PI3K, Akt/protein kinase B, Rap1b, and G protein-gated inwardly rectifying potassium channels (GIRKs).³³

The PI3K isoforms that function downstream of G β γ in platelets are PI3K γ and PI3K β . The two isoforms seem to function in distinct but complementary roles leading to platelet aggregation and release. PI3K γ plays a role in enhancing granule secretion while PI3K β is necessary for sustaining platelet aggregation and formation of stable thrombi.²⁹ Akt/protein kinase B activation is thought to be mediated via PI3K γ . Deficiency of Akt/protein kinase B in mice results in impaired platelet aggregation and release, and like PI3K β , it is required for formation of stable thrombi.⁶⁵ PI3K γ and PI3K β , via generation of phosphatidylinositol (3,4,5) trisphosphate (PIP₃), activate Rap1b, a critically important step in mediating the α IIb β 3 conformational change required for fibrinogen binding and platelet aggregation.³⁷

GIRK channels containing GIRK1 and GIRK4 subunits are involved in the activation of Src kinases. Channel activation is coupled to G β γ and ADP occupancy of the P2Y₁₂ receptor. Src kinases in turn phosphorylate and activate cytoplasmic phospholipase A2 (cPLA₂).⁵⁶ P2Y₁ activation and signaling via G α q as well as signaling through α IIb β 3 are also necessary for cPLA₂ activation.³¹ When phosphorylated, cPLA₂ is translocated from the cytoplasm to membranes where it functions to release membrane-bound arachidonic acid leading to the generation of thromboxane. Human patients with congenital P2Y₁₂ deficiency and P2Y₁₂ knock-out mice have prolonged bleeding times and exhibit bleeding, illustrating the importance of this receptor in hemostasis. The absent aggregation response of P2Y₁₂ deficient platelets can be restored if platelets are activated concomitantly with ADP and epinephrine, presumably due to epinephrine being negatively coupled to adenylyl cyclase in a manner similar to P2Y₁₂.¹⁰

The P2X₁ receptor is an ATP receptor that is antagonized by ADP; early studies demonstrating P2X₁ receptor responses to ADP were later shown to be a result of ATP contamination of ADP preparations.³⁸ P2X₁ activation is associated with the change in conformation of platelets from discs to spheres with the rapidly reversible formation of multiple short pseudopodia.^{50,51} ATP

binding to P2X₁ is associated with a rapid, reversible calcium influx followed by rapid desensitization of the receptor.⁴² Calcium influx is thought to activate MLC-kinase which is responsible for mediating the shape change response. P2X₁ activation also results in centralization of granules without granule release and formation of platelet microaggregates.^{21,61} Microaggregate formation is likely a result of calcium influx coupled with protein kinase C activation leading to Erk2 activation.³³ Like the P2Y₁₂ receptor, P2X₁ also participates in amplification of platelet responses to low concentrations of other agonists, including ADP, collagen, thrombin, and thromboxane.^{21,22,42} Dense granule release of ATP induced by low concentrations of agonists triggers P2X₁-mediated calcium influx resulting in reinforcement and amplification of platelet activation. P2X₁ also seems to be essential in thrombus formation and growth at sites of injury in vessels where high shear forces are generated.²⁶

Collagen Receptors

Platelets have two receptors that are primarily responsible for direct response to collagen; glycoprotein VI (GPVI) and integrin α 2 β 1.⁴⁹ (Fig. 76.2) The platelet glycoprotein Ib-IX-V complex, which interacts with collagen indirectly, is critically important in initiating platelet contact with collagen under high shear rate conditions. The glycoprotein Ib-IX-V complex mediates transient arrest of platelets from flowing blood and weak tethering of platelets on exposed subendothelial surfaces through the binding of von Willebrand factor (VWF) A1 domain to the GPIb α region of the receptor complex. As tethered platelets roll along exposed subendothelium they encounter collagen fibrils, which bind to glycoprotein VI. This leads to inside-out signaling and activation of integrins α 2 β 1 and α IIb β 3 and the release of ADP and thromboxane formation which in turn reinforce GPVI interactions.⁴¹ Activated α 2 β 1, transformed to a high affinity state, binds tightly to specific sequences in collagen, allowing firm platelet adhesion and spreading.³⁴ Activated α IIb β 3, also transformed to a high affinity state, reinforces firm platelet adhesion and supports platelet aggregation.⁵³

Integrin α 2 β 1 and GPVI are thought to signal through a shared network of signal transduction proteins. The shared signaling pathway possibly explains why the absence of either GPVI or integrin α 2 β 1 does not result in a significant bleeding diathesis.

Glycoprotein VI is a member of the immunoglobulin superfamily Type I transmembrane proteins; it is non-covalently associated with the Fc receptor gamma-chain (FcR γ -chain). The FcR γ -chain has an immunoreceptor tyrosine-based activation motif (ITAM). When stimulated with collagen, the FcR γ -chain is tyrosine phosphorylated by the Src kinases, Lyn and Fyn.²³ This leads to the recruitment and activation of Syk tyrosine kinases which lead to the phosphorylation and activation of other signaling molecules.

Adaptor molecules such as linker for activation of T cells (LAT) and Src homology domain-containing

leukocyte phosphoprotein of 76 kDa (SLP-76) coordinate assembly of a multiprotein signaling complex necessary for activation of phospholipase C γ 2 (PLC γ 2) and phosphoinositide-3-kinase (PI3K). LAT is a membrane associated adapter molecule that is anchored to lipid rafts in platelet membranes.⁴⁷ Activation of PLC γ 2 leads to cleavage of phosphatidylinositol 4,5 bisphosphate into inositol 1,4,5-triphosphate (IP3) and 1,2 diacylglycerol (DAG). IP3 mediates the release of calcium from stores within the dense tubular system. DAG and calcium bind to calcium diacylglycerol guanine nucleotide exchange factor I (CaDAG-GEFI) resulting in activation of PKC and activation of Rap1b. Rap1b activation plays a role in inducing the change in conformation of α IIb β 3 to a high affinity state necessary for binding of fibrinogen and platelet aggregation. Although GPIb-IX-V also stimulates PLC γ 2 activation, the tyrosine phosphorylation pattern is distinct from that occurring via GPVI, resulting in weaker PLC γ 2 activation and diminished phospholipase activity.⁶⁰

Collagen induced platelet aggregation is largely dependent on ADP signaling through P2Y12. Thromboxane A2 (TXA) generation and resulting platelet aggregation and release result in signaling through the P2Y12 receptor by secreted ADP and a positive feedback on platelet secretion through the PI3K pathway.⁴⁴

Thromboxane Receptors

Thromboxane A2 (TXA) and prostaglandin H2 interact with the same receptors. Human platelets have two distinct thromboxane A2-prostaglandin H2 receptors, TP α and TP β , that are products of alternative splicing. These receptors differ only in their C-terminal or tail domains.²⁸ TP α is a high-affinity binding site for thromboxane and mediates platelet shape change and intracellular calcium movement. TP α couples positively to adenylate cyclase via Gq and activates PLC β which hydrolyzes PIP2, resulting in generation of IP3 and DAG. TP β is a low-affinity binding site and mediates platelet aggregation and secretion. TP β couples negatively to adenylate cyclase via Gi and inhibits cAMP formation. Platelets obtained from most dogs have a weak arachidonic acid (AA) response characterized by shape change and reversible platelet aggregation without granule release.³² Addition of epinephrine prior to AA corrects the impaired aggregation and release response.²⁰ Canine platelets likely only possess TP α receptors based on the absence within the dog genome of the coding region necessary for synthesizing the alternative C-terminal domain for TP β . This may partially explain the minimal response of the majority of canine platelets to AA stimulation *in vitro*. Epinephrine, by linking to Gi, may mimic the effects of TP β thus allowing for full platelet aggregation and release. A bovine TP α receptor has been cloned and characterized.³⁹

Platelets generate TXA from AA. AA is liberated from dense tubular membrane phospholipids by the action of phospholipase A2 in response to agonist stimulation. Cytosolic phospholipase A2 (cPLA2) is phos-

phorylated during thrombin, ADP, and collagen induced platelet activation. Phosphorylation of cPLA2, in combination with a rise in intracellular calcium, results in cPLA2 translocation to the membrane where it releases arachidonic acid from the sn-2 position of membrane-bound phospholipids. Arachidonic acid is converted sequentially to prostaglandin G2 (PGG2), prostaglandin H2 (PGH2), and TXA via cyclooxygenase-1, prostaglandin hydroperoxidase, and thromboxane synthetase. The resulting thromboxane generation results in platelet aggregation and dense granule release.

TXA synthase inhibitors do not inhibit platelet reactivity due to the enhanced production of prostaglandin H2 under these conditions and the ability of prostaglandin H2 to activate the receptor.⁶³

Thrombin Receptors

Platelet thrombin receptors include the glycoprotein complex Ib-IX-V and protease activated receptors (PARs). The Ib α subunit of the Ib-IX-V complex has a thrombin binding site that overlaps with the binding site for VWF.⁴⁸ The GP V subunit is cleaved and removed from the complex by thrombin during platelet activation. GP V is thought to negatively modulate platelet activation; cleavage of GP V is a necessary first step prior to binding of thrombin to Ib α . Thrombin binding to Ib α mediates platelet activation via induction of ADP release and signaling through P2Y12. Ib α is also thought to act as a cofactor in the activation of PAR1 by thrombin.¹⁷ Thrombin bound to Ib α is protected from inactivation by antithrombin.¹⁸

Protease activated receptors (PARs) are G-protein coupled receptors that are characterized by the presence of a tethered ligand (Fig. 76.2). Protease activated receptors are activated when enzymes cleave at a specific site near the N-terminal exodomain resulting in formation of a tethered peptide sequence that can in turn bind to a G-protein coupled receptor on the same molecule. Peptide sequences that match the tethered ligands generated by thrombin cleavage, thrombin receptor activation peptides or TRAPs, have been used with varying success *in vitro* to activate platelets.^{9,11} In most species, TRAPs are not as effective as the comparable tethered ligand in inducing platelet aggregation or release. Mammalian platelets contain three PAR types that vary with species. Human platelets have PAR1 and PAR4 while mouse platelets signal through PAR3 and PAR4. PAR1 couplings to Gq, G13, and Giz are thought to be of most importance in mediating thrombin effects on platelets.¹²

The PI3 kinase pathway is considered to be of importance in stabilizing thrombin-induced platelet aggregates, although thrombin can activate platelets independently of Gi activation. On human platelets PAR1 binding results in a rapid spike in calcium influx while PAR4 binding is associated with a slower and sustained calcium influx that is considered vital to eliciting secondary signals necessary for complete platelet activation.¹³ Canine platelet PARs have not been fully

evaluated; however, it is likely that canine platelets are similar to human platelets and possess PAR1 and PAR4 receptor subtypes.⁵ Canine platelets do not respond to TRAPs designed from human or canine sequence¹⁹ (M.K. Boudreaux, personal observation). There is genomic evidence for PAR3 receptor subtypes on both human and canine platelets; however, PAR1 and PAR4 receptors are considered to be of primary importance in mediating thrombin-induced platelet activation. Mouse platelets do not possess PAR1. PAR3 on mouse platelets acts as a cofactor in supporting cleavage and activation of PAR4. PAR3 alone is unable to mediate signaling leading to platelet activation but rather acts as a cofactor for promoting PAR4 cleavage in the presence of low thrombin concentrations.⁴⁰

PLATELET-LEUKOCYTE INTERACTIONS

The GPIIb α subunit of platelet glycoprotein complex Ib-IX-V is a ligand for endothelial P-selectin, as well as VWF, both of which translocate from Weibel-Palade bodies to endothelial surfaces in response to stimuli such as thrombin and histamine.^{2,52,67} The binding site on GPIIb α for P-selectin is very similar to the binding site of P-selectin glycoprotein ligand (PSGL-1) which allows leukocytes to roll along the vascular endothelium. Both GPIIb α and PSGL-1 are membrane mucins and possess a heavily O-glycosylated region that separates the ligand-binding region from the plasma membrane.³⁶ Both receptors also contain an acidic region immediately amino-terminal to the mucin-like region that is important for ligand binding.³⁵ Unlike PSGL-1, which requires extensive post-translational modification, including fucosylation of side chains in order to interact with P-selectin, GPIIb α is capable of binding to endothelial P-selectin without modifications.

GPIIb α can also bind to integrin α M β 2 (Mac-1)⁵⁷ likely via the I domain within the α M subunit which closely resembles the A1 domain of VWF. This interaction, which requires conversion of Mac-1 to the ligand-binding competent form, may contribute to neutrophil attachment to the endothelium or subendothelium at sites of endothelial activation or injury. Platelet P-selectin can bind to PSGL-1 on neutrophils, monocytes, and T-cells providing a mechanism for initial adhesion of leukocytes to platelets. Firm platelet-leukocyte adhesion is mediated by binding of Mac-1 to fibrinogen bound to the surface of activated platelets.

Firm leukocyte-platelet interactions can also be mediated by intracellular adhesion molecule 2 (ICAM-2) on platelets which can bind to α L β 2 (LFA-1; CD11a/CD18) on leukocytes. Platelets can also release CD40L which binds to CD40 on vascular endothelial cells resulting in chemokine secretion and up-regulation of adhesion molecules.²⁷ Platelets also secrete IL-1 β which can induce endothelial cell secretion of IL-6, CXCL8, and CCL2 and up-regulation of adhesion molecules such as E-selectin, VCAM-1, ICAM-1, and α v β 3.²⁴ All of these events link platelets to the inflammatory process.

PROCOAGULANT ACTIVITY

Platelet activation triggers significant remodeling of the platelet cell surface. This is associated with the activation and clustering of receptors for adhesion molecules, receptor mediated outside-in signaling, relocation and binding of proteins and other biomolecules from the cytosol to membrane surfaces, secretory organelle movement and fusion, and a dramatic redistribution of membrane phospholipids and formation and release of platelet membrane derived microparticles.

Mammalian cells including platelets exhibit an asymmetric distribution of aminophospholipids. In non-activated platelets, negatively charged aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) are located primarily on the inner leaflet of the plasma membrane. An aminophospholipid translocase is responsible for transporting PS and PE to the inner membrane leaflet and maintaining asymmetry. When platelets are activated by collagen or thrombin alone or in combination, PS is rapidly externalized and maintained on the outer leaflet of the membrane (Fig. 76.3). This redistribution is also accompanied by the release of PS-enriched procoagulant microparticles. Phosphatidylserine is also externalized in cells exposed to apoptotic triggers.

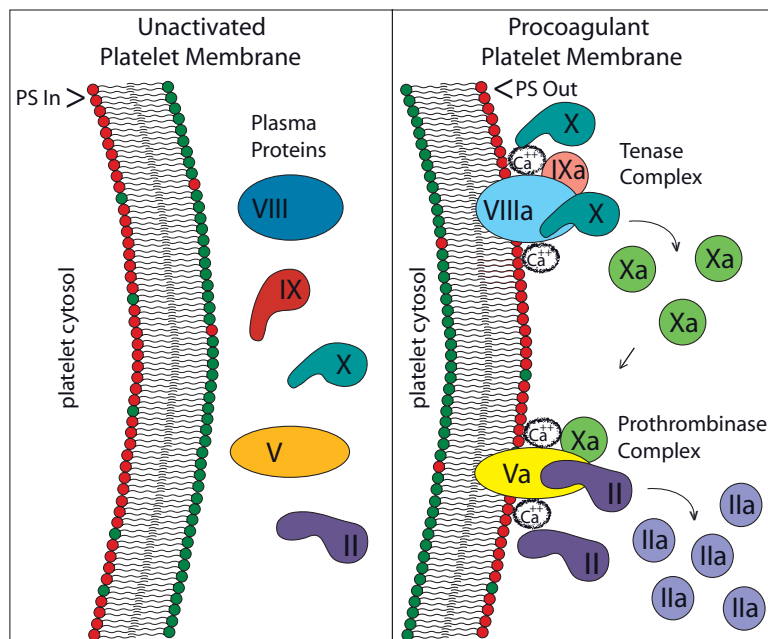
Activation of the lipid transporter protein referred to as "scramblase" functions to move phospholipids regardless of charge in a bidirectional fashion resulting in loss of membrane asymmetry and rapid externalization of PS. Phosphatidylserine remains externalized due to the inhibition of aminophospholipid translocase when "scramblase" is activated.

The presence of PS on the surface of platelets is essential for expression of platelet procoagulant activity. Rare bleeding disorders associated with a deficiency of platelet procoagulant activity have been reported in humans and dogs.^{7,62} Phosphatidylserine facilitates the binding of coagulation proteins, which when anchored to PS and the activated platelet surface form procoagulant reaction complexes. These include the extrinsic tenase (factor VIIa + tissue factor (TF) + factor X), intrinsic tenase (factor X + factor IXa + factor VIIIa) complexes and the prothrombinase complex (factor Va + factor Xa and factor II). Once assembled the complexes allow for protein associations, which improve catalytic efficiency of the reactions. For example the prothrombinase complex is about 300,000 times more efficient than Xa alone in generation of thrombin from prothrombin, and the intrinsic tenase complex is more than 100,000 times more active than factor IXa alone as an activator of factor X.

The assembly of reaction complexes on the PS enriched surface of activated platelets serves to localize and concentrate procoagulant reaction products at sites of injury. Since AT preferentially targets uncomplexed reaction products for neutralization, the anchored reaction products, including FXa and thrombin, are protected from neutralization and inhibition by AT.

Lipid scrambling is associated with formation of PS-enriched procoagulant membrane microparticles which

FIGURE 76.3 Signaling pathways activated by collagen and thrombin trigger externalization of phosphatidylserine (PS). When PS is externalized, specific coagulation proteins can bind to the platelet surface. The platelet then acts as a scaffold for assembly of the tenase and prothrombinase complexes, which accelerate thrombin (II_a) generation and clot formation at the site of vessel injury.



are shed from the plasma membrane. High cytoplasmic calcium concentrations are thought to activate lipid scramblase and inhibit aminophospholipid translocase, thus leading to expression of PS on the surface of activated platelets. Other studies have shown that expression of procoagulant activity is dependent on a change in intracellular pH and Na influx which occurs with activation of the Na/H exchanger (NHE1), and potentially other mechanisms, in human and porcine platelets.⁵⁴ NHE1 can be phosphorylated and activated by PKC. NHE1 also has two PIP₂-binding domains which are required for optimal activation, suggesting that PIP₂ may play a role in mediating PS exposure in activated platelets.^{1,8} Studies have shown that only small populations of platelets express PS upon activation with thrombin or collagen. Combining the two agonists results in an increase in this fraction of platelets and an overall increased PS signal when assessed by flow cytometry.⁶⁴

Activated platelets have been shown to be capable of synthesizing and expressing tissue factor on their surface which contributes to the prothrombinase function of platelets. Platelets possess pre-mRNA transcripts which are spliced and translated into protein upon platelet activation. Cdc2-like kinase dependent splicing is thought to play a role in this mechanism.⁵⁵

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Platelet Kinetics and Laboratory Evaluation of Thrombocytopenia

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Platelet Kinetics

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Acronyms and Abbreviations

APTT, activated partial thromboplastin time; BFU-Meg, burst forming unit megakaryocyte; CBC, complete blood count; CFU-Meg, colony forming unit megakaryocyte; CMP, common myeloid progenitor cell; DIC, disseminated intravascular coagulation; DMS, demarcation membrane system; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FDP, fibrin-fibrinogen degradation product; FXII, factor XII; GM-CSF, granulocyte-monocyte colony stimulating factor; HUS, hemolytic uremic syndrome; IL, interleukin; MPC, mean platelet component concentration; MPV, mean platelet volume; OSPT, one-stage prothrombin time; PCDW, platelet component concentration distribution width; PCT, plateletcrit; PDW, platelet distribution width; PT, prothrombin time; SDF-1, stromal cell-derived factor-1; TTP, thrombocytopenic thrombotic purpura; VWF, von Willebrand factor.

PLATELET KINETICS

Megakaryocytopoiesis and Platelet Production

Platelets are the second most numerous circulating cell in blood and are essential for coagulation, maintenance of vascular integrity, and control of hemostasis. In mammals, these small, anucleated cells originate in the bone marrow from megakaryocytes.

Megakaryocytopoiesis progresses through many stages starting with a pluripotential stem cell and proceeds through subsequent development of committed precursors. The hematopoietic pluripotential stem cells and early megakaryocyte progenitor cells are positive for CD34 and have low expressions of CD41.^{42,49} Cells

that become committed to the megakaryocyte lineage acquire the expression of CD61 and CD42, and expression of CD41 increases.^{42,49} From the hematopoietic stem cell, megakaryocytopoiesis progresses through the common myeloid progenitor cell (CMP), followed by a bipotential progenitor cell for erythroid and megakaryocytic cells.^{20,41,58} The next stage, a primitive megakaryocytic burst-forming unit (BFU-Meg), has a high proliferative capacity and can produce anywhere from 100 to 500 megakaryocytes per colony in a 1 week time period.^{11,42} Following the BFU-Meg, the more mature megakaryocytic colony-forming unit (CFU-Meg) gives rise to approximately 3–50 megakaryocytes per colony. The BFU-Meg and CFU-Meg stages express CD34, CD33, and CD41. Later stages gain CD61 expression

followed by CD42 and CD36. The first morphologically recognizable cell of the megakaryocyte lineage in marrow is the promegakaryoblast. Before this stage, the morphology of the earlier progenitors resembles a small lymphocyte. The promegakaryoblast has a high N:C ratio, dark blue cytoplasm, and is approximately 10 μm in diameter.^{42,103} The megakaryoblast has a kidney-bean shaped nucleus, dark blue cytoplasm and is approximately 15–50 μm in diameter. As the later stages of megakaryocyte precursors undergo endomitosis, the cells lose CD34 expression, and increase ploidy, cell size and volume. Megakaryocytes undergo DNA duplication as low as 2N and can attain ploidy as high as 128N, with the modal ploidy in most mammals being 16N.¹³ Mature megakaryocytes are the largest hematopoietic cells in marrow, reaching diameters as high as 150 μm . The higher ploidy corresponds to an increased cell volume, and as a result, a higher number of platelets that are produced.¹³

Each megakaryocyte is capable of generating several thousand platelets. In humans, daily platelet production is estimated to be approximately 35,000 \pm 4,300 platelets/ μL or 1×10^{11} total platelets.^{13,45,49} It takes 4–5 days to complete the sequence of megakaryocyte development to the release of new platelets.⁴⁵ Mature megakaryocytes produce platelets by cytoplasmic fragmentation through regulated and dynamic processes that involve the invagination of the megakaryocyte plasma membrane which forms the demarcation membrane system (DMS) and the association of the DMS with microtubules and an actin/myosin complex.^{69,74} The association with microtubules causes evagination of the DMS and elongation of pseudopodal extensions that result in proplatelet formation.^{13,69} The actin/myosin complex is thought to be important in proplatelet formation and the separation of platelets from proplatelets.⁸² In marrow, mature megakaryocytes migrate so that they are closely positioned near endothelial cells. This allows the long proplatelet pseudopods from the megakaryocyte to extend into marrow sinusoids and also allows the megakaryocyte to enter the peripheral circulation. The final process of platelet shedding has to occur in the circulation to prevent platelet trapping within the marrow.^{13,95} This latter observation may explain, in part, why megakaryocytes are often found in pulmonary circulation where significant platelet shedding occurs.⁹⁸

Thrombopoietin is the major cytokine that regulates all stages of megakaryocyte and platelet production. In addition to thrombopoietin, megakaryocytes and platelet production are regulated by several growth factors and cytokines. Many of these affect hematopoiesis in general, or work synergistically with thrombopoietin. Megakaryocyte progenitors are stimulated by interleukin (IL)-3, IL-6, IL-11, IL-12, granulocyte-monocyte colony stimulating factor (GM-CSF), and erythropoietin.³² Additional factors important in thrombopoiesis include IL-1, stem cell factor, and leukemia inhibitory factor.^{13,32} Stromal cell-derived factor 1 (SDF-1) is thought to play a role in megakaryocyte migration.³⁴

Platelet production is regulated by total platelet mass rather than platelet numbers. In situations of increased demand, platelet production can increase as much as 20-fold or higher.⁴⁹ In health, platelet numbers are relatively stable and constant, and the thrombopoietin-thrombopoietin receptor system is responsible for maintaining platelet mass. Thrombopoietin is secreted constitutively from the liver and to a lesser extent from the kidney. High affinity thrombopoietin receptors, present on platelet surfaces, bind thrombopoietin, which is then internalized and degraded.^{48,52} In thrombocytopenic states, a higher level of free thrombopoietin is available to stimulate platelet production.

Circulating Lifespan and Senescence

Platelet numbers remain fairly constant within a species, but platelet numbers vary widely between different species. In addition, approximately 30% of the platelet circulating mass is transiently compartmentalized in the spleen for a short period of time in a resting individual. In health, there is a steady state between the number of platelets produced and platelets destroyed. Platelets circulate for approximately 5–9 days in most mammalian species. As platelets age, they are removed from circulation by macrophages in the spleen and liver. Signals involving platelet senescence are not completely characterized.⁵⁹ Exposure of phosphatidylserine on the outer platelet membrane, damaged or denatured platelet glycoproteins and proteoglycans, and gradual loss of platelet fragments or microparticles are thought to play important roles in platelet attrition.⁵⁹ Phosphatidylserine may induce macrophage phagocytosis directly or through bridging proteins that bind macrophage and phosphatidylserine receptors.^{39,59} Similar to erythrocyte senescence, glycoproteins on the platelet surface may become denatured or damaged through changes in glycosylation or through the exposure of neoepitopes that are recognized by naturally occurring antibodies.⁵⁹ A smaller percentage of platelets are continually removed from circulation because of their role in the maintenance of endothelial integrity.^{30,59}

Non-mammalian Species

The cellular equivalent of platelets in birds, reptiles, amphibians, and fish is the thrombocyte, which is a small, round, oval, or spindle nucleated cell. Similar to mammals, the primary function of thrombocytes is hemostasis and coagulation, and thrombocytes are the second most prevalent circulating cell in blood after erythrocytes. Avian thrombocytes may also be important in innate immunity since these cells have phagocytic capability and can remove foreign material from blood.³³ In addition to hemostasis, reptilian thrombocytes play a role in wound healing.⁹³

Thrombopoiesis is similar in avian and reptilian species in that thrombocytes originate in the bone marrow from a distinct mononuclear cell. The stages in thrombopoiesis include thromboblats, immature

thrombocytes (early, mid, and late stages), and mature thrombocytes. In general, as thrombocytes develop, they decrease in size, the cytoplasm changes from basophilic to pale blue or colorless, and the cell and nuclear shape change from round to oval.

Thrombocyte numbers in most avian species range between 20,000 and 30,000/ μL or approximately 10–15 thrombocytes per 1000 erythrocytes; 1–2 thrombocytes per monolayer area under oil immersion (100 \times) is considered normal or adequate in most birds.^{10,25} In normal reptiles, thrombocyte numbers in blood range between 25 and 350 thrombocytes per 100 leukocytes.⁹³ Numbers of thrombocytes in fish blood range from 60,000 to 70,000/ μL .⁷⁸ Manual counts for thrombocytes in fish can be performed using a hemacytometer, but given the tendency to clump, accurate counts are difficult to obtain. Thrombocytes of certain fish species do not appear to have phagocytic ability.⁷⁸

BASIC MECHANISMS OF THROMBOCYTOPENIA

Thrombocytopenia is defined as a decrease in circulating platelets and is the most common acquired hemo-

static disorder in veterinary and human medicine. Thrombocytopenia results from one or a combination of the following basic mechanisms: decreased or defective platelet production, increased peripheral platelet loss or consumption, increased platelet destruction, or abnormal distribution. To understand a diagnostic approach (Fig. 77.1) to thrombocytopenia, a basic understanding of the mechanisms leading to thrombocytopenia is useful, and an overview is discussed below. The pathogenesis of thrombocytopenia is covered in more detail in Chapters 78 and 79.

Decreased or Defective Platelet Production

Platelets originate from megakaryocytes found in the bone marrow and to a lesser extent in the lung. Causes of decreased platelet production that result in thrombocytopenia can specifically target the megakaryocyte, but more frequently, other hematopoietic cells are also affected.

Although rare, acquired megakaryocyte hypoplasia or aplasia has been reported in dogs and cats, and probably has an immune-mediated component.^{27,54,65} General etiologies for bone marrow disease leading to hypopla-

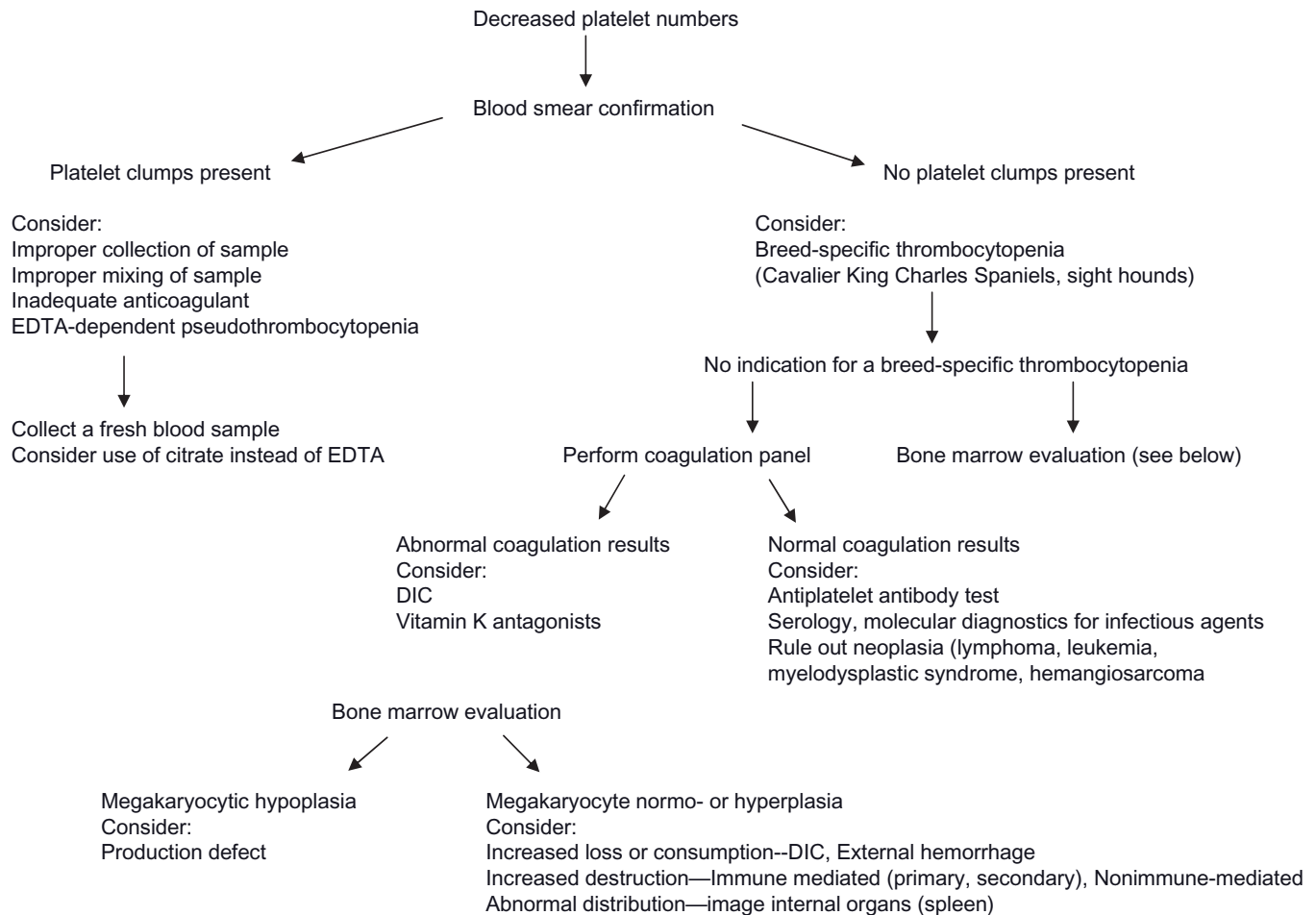


FIGURE 77.1 Diagnostic approach to thrombocytopenia.

sia of one or more hematopoietic cell lines include drugs, chemicals, or toxins that have cytotoxic effects or produce idiosyncratic reactions; irradiation resulting in cell death and marrow suppression; infection with certain viruses (canine parvovirus, feline leukemia virus, feline immunodeficiency virus, equine infectious anemia virus, bovine viral diarrhea virus, African swine fever virus) or rickettsial agents; myelophthisis from primary or metastatic neoplasia or myelofibrosis; or myelonecrosis.

Increased Platelet Loss or Consumption

Thrombocytopenia secondary to rapid, increased loss of platelets may occur with massive trauma, extensive external hemorrhage, or exchange blood transfusion. In situations of trauma or external hemorrhage, thrombocytopenia is generally mild to moderate, transient, and usually reversible without specific treatment.⁸⁵

Accelerated consumption of platelets can be brought on by widespread activation of the coagulation system or endothelial damage. Disseminated intravascular coagulation (DIC), thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS) are complex syndromes characterized by widespread consumption of platelets, resulting in moderate to significant thrombocytopenia. Disseminated intravascular coagulation is a common complication seen in both veterinary and human medicine. Thrombotic thrombocytopenic purpura and HUS occur in humans but are rare in veterinary species. A syndrome resembling HUS has been reported in dogs, cats, horses and a heifer.^{2,14,21,22,40,56,63,79} Animal models of TTP and HUS have been described.^{77,81,102}

Disseminated Intravascular Coagulation

Disseminated intravascular coagulation occurs secondarily to a wide variety of insults and diseases; DIC can be acute, subacute, or chronic and is characterized clinically by hemorrhage and microthrombosis. The pathophysiology of DIC is discussed in more detail in Chapter 88.

Common disorders associated with DIC include vascular damage, septicemia with release of bacterial endotoxins, release of tissue thromboplastin from necrotic or malignant tissue, or release of other procoagulant proteins. Approximately 65% of DIC cases in humans occur secondarily to infection; bacterial infection is the most common.¹⁰⁷ In Gram-negative infections, bacterial release of endotoxin (lipopolysaccharide) causes simultaneous activation of the coagulation system and inhibition of coagulation control mechanisms. Endotoxin stimulates intrinsic coagulation directly by activation of factor XII (FXII) or indirectly by activation of FXII via endothelial damage. Endotoxin stimulates the extrinsic coagulation pathway by causing generation and increased surface expression of thromboplastins or tissue factor by inflammatory cells, primarily monocytes. Activated monocytes release IL-1 and tumor

necrosis factor alpha (TNF- α), which decreases endothelial expression of thrombomodulin and prevents activation of protein C, thus impairing an important coagulation inhibitory pathway.

Thrombotic Thrombocytopenic Purpura and Hemolytic Uremic Syndrome

In humans, the thrombotic microangiopathies are exemplified by two syndromes, TTP and HUS. The pathogenesis of both is heterogeneous and often the origin is unknown; many consider them to be different expressions of the same disease mechanism. Thrombotic thrombocytopenic purpura and HUS may occur either as a primary condition or as a secondary complication. Most cases of HUS occur in childhood after bloody diarrhea caused by *Shigella dysenteriae* serotype I or various *Escherichia coli* serotypes. The classical triad of TTP is characterized by severe thrombocytopenia, intravascular hemolysis with schistocytes, and neurologic symptoms. Severe renal dysfunction is also a prominent feature of HUS.

The thrombotic microangiopathies are characterized by endothelial damage and platelet aggregation with resultant thrombocytopenia, hemolytic anemia, and thrombosis. High plasma concentrations of thrombomodulin, tissue plasminogen activator, and von Willebrand factor (VWF) are observed in many TTP patients.⁹⁴ Plasminogen activator inhibitor type I and urine endothelin concentrations are increased in HUS patients.^{5,87} Concentrations return to normal when patients enter remissions.

Platelet aggregation may be caused by the presence in plasma of abnormally large VWF molecules that bind platelets and cause aggregation and activation much more actively than smaller VWF molecules. In TTP, endothelial damage may cause leakage of stored large VWF multimers into subendothelial tissue and plasma. Normal cleavage and clearance of these large VWF multimers is limited, causing increased concentrations and extensive platelet aggregation.⁶¹ This extensive, systemic platelet aggregation causes platelet consumption and moderate to significant thrombocytopenia. Hemolytic uremic syndrome and TTP are discussed in more detail in Chapter 79.

Platelet Destruction: Immune Mediated, Non-immune Mediated, and Complex Mechanisms

Immune-mediated thrombocytopenia is covered in Chapter 78. Briefly, immune-mediated destruction of platelets may be primary (idiopathic) or may occur in association with infectious agents, neoplasia, drugs, autoimmune, isoimmune, or neonatal-immune diseases. A diagnosis of idiopathic or primary immune-mediated thrombocytopenia is made after all other potential causes have been eliminated.

Immune destruction of platelet occurs when circulating platelets coated with antibody, antigen-antibody complexes or complement are phagocytized by macrophages in the spleen, liver, and bone marrow. The result

is decreased platelet survival and lifespan. The spleen is the largest lymphoid organ, the major site of antibody production and platelet removal.

Platelet aggregation, phagocytosis, or lysis resulting in platelet destruction and thrombocytopenia can occur independently of immune-mediated events. Non-immune-mediated platelet destruction occurs in some acute bacterial and viral infections and in cardiovascular disease. Thrombocytopenia occurring from venomous snakebites may be secondary to DIC or may result from direct aggregation of platelets. Platelet activation with thrombocytopenia is associated with severe and extensive burns.

Platelet destruction in bacterial infections can occur as a result of platelet adherence or aggregation to activated monocytes or neutrophils. In Gram-negative infections, monocytes stimulated by endotoxin express tissue factor on their surface and generate thrombin.⁸³ Thrombin, a potent platelet agonist, causes platelet activation and aggregation. Platelets adhere to monocytes and are phagocytized. Neutrophil stimulation is not believed to be a major cause of platelet removal and is probably more important with regard to platelet function and thrombus formation.³⁶ Exotoxins released from Gram-positive bacteria may directly damage platelets and contribute to thrombocytopenia.^{31,47,76,97}

The pathogenesis of thrombocytopenia associated with acute viral infections is often multifactorial, even though one mechanism may predominate. Direct platelet damage or lysis is one proposed mechanism of viral-associated thrombocytopenia. Myxovirus infections, including those due to Newcastle disease virus and influenza, decrease platelet survival by removal of platelet membrane sialic acid residues by viral neuraminidase.^{96,99} Other viruses in which platelet damage is suspected include bovine viral diarrhea virus and hog cholera virus.^{16,104}

In humans, platelets can be damaged and destroyed by mechanical means in arterial disease with roughened endothelial surfaces and narrowed microcirculation, stenotic or prosthetic heart valves, or cardiac by-pass surgery.^{6,17,29} Turbulent circulation causes membrane damage and microparticle formation.²⁸ Interactions of platelets with altered or damaged endothelial surfaces cause extensive platelet activation, clumping, and removal of platelets by the mononuclear phagocyte system.⁶ This may also occur in viral infections that infect or alter endothelial cells. *In vitro*, virally transformed endothelial cells show a significant increase in platelet adherence.¹⁸

Many of the rickettsial diseases are associated with thrombocytopenia. Several mechanisms contribute to thrombocytopenia, including immune-mediated platelet destruction, direct damage, vasculitis, and platelet production deficits. Disseminated intravascular coagulation may occur as a secondary complication.

Abnormal Platelet Distribution

In health approximately 30–40% of the total circulating platelet pool may be stored in the spleen, which is

referred to as physiologic platelet sequestration. The liver and bone marrow are additional tissue sites of platelet sequestration.³ Hypersplenism is a pathologic condition in which as much as 90% of circulating platelets become sequestered predominately in the spleen. Hypersplenism is characterized by the presence of one or more cytopenias with corresponding bone marrow hyperplasia and significant splenomegaly. Splenic size is probably the most important factor that determines the degree of thrombocytopenia; the spleen must be significantly enlarged to cause a severe decrease in circulating platelets.⁷¹ Although hypersplenism is characterized by splenomegaly, the presence of splenomegaly does not always signify hypersplenism.

Thrombocytopenia that results from hypersplenism can be thought of as a displacement of the majority of platelets from peripheral circulation into a reversible, but slowly exchanging, splenic pool.²⁹ Thrombocytopenia secondary to splenic pooling is different from that seen with idiopathic thrombocytopenic purpura in which there is active removal of platelets by the splenic macrophages.²⁹ In hypersplenism, thrombocytopenia is evident on a complete blood count (CBC), but the total numbers of platelets and platelet mass are actually normal. Platelet survival is usually normal, as is platelet production.^{3,37}

Hypersplenism is rare in animals but a few suspected cases have been described.^{44,51} All other causes of splenomegaly should be ruled out before a diagnosis of primary hypersplenism is made. Recommended treatment in these cases is splenectomy.

Severe hypothermia adversely affects platelet morphology and can cause a mild transient thrombocytopenia owing to pooling of platelets in the spleen.⁴⁴ Transient thrombocytopenia can also occur with endotoxemia and hypotension.

Pseudothrombocytopenia

Pseudothrombocytopenia (artifactual or spurious thrombocytopenia) is an *in vitro* phenomenon resulting from platelet clumping secondary to poor or difficult venipuncture and subsequent platelet activation or exposure of the blood to certain anticoagulants. Feline platelets are especially prone to activation during blood collection. Pseudothrombocytopenia can occur with overfilling of a blood collection vacutainer tube and subsequent improper or inadequate sample mixing.⁷²

Uncommonly, *in vitro* clumping occurs in certain individuals when blood is collected into anticoagulants. This has been seen most commonly with ethylenediaminetetraacetic acid (EDTA) and rarely with other anticoagulants. EDTA-dependent pseudothrombocytopenia has been reported in humans, a horse, a miniature pig, and a dog.^{30,38,75,105} Although there may be several factors, the leading mechanism for EDTA-dependent pseudothrombocytopenia involves autoantibody-mediated platelet agglutination. Hidden epitopes become exposed within the platelet IIb-IIIa complexes as a result of complex destabilization occurring with calcium removal from calcium binding domains.^{8,12,23}

Rarely, platelet clumping can occur with exposure to anticoagulants containing citrate, oxalate, or heparin.^{68,70,86}

Accurate automated platelet counts in cats are often difficult to obtain due to clumping. Feline platelets are more reactive than those of other species. Several factors unique to feline platelets may be involved, including a larger platelet size, a higher concentration of serotonin, irreversible aggregation and granule release when exposed to serotonin, and irreversible aggregation in response to low concentrations of ADP.^{9,57,73} Because of their nature and small size and the fact that many cats resist handling and restraint in unfamiliar settings, venipuncture is often challenging, which may increase the likelihood of platelet activation *in vitro*.⁶⁶

CLINICAL SIGNS OF THROMBOCYTOPENIA AND RISK OF HEMORRHAGE

Clinical signs leading one to suspect thrombocytopenia include petechiation or ecchymosis in tissues or mucosal membranes, epistaxis, melena, hematochezia, hematuria, prolonged bleeding after venipuncture, or retinal hemorrhage or hyphema. However, clinical signs of bleeding are infrequently seen. More commonly, thrombocytopenia is usually identified on a routine CBC. Animals having a low platelet count may exhibit no clinical signs or present for lethargy, weakness, fever, or other signs related to an underlying disease process. Despite the observation that hemorrhage is not a consistent feature, thrombocytopenic animals are at an increased risk of bleeding.

Hemorrhage solely caused by thrombocytopenia does not usually occur until peripheral platelet numbers are severely decreased (<50,000 platelets/ μ L). Animals that have platelet counts of $\leq 10,000/\mu$ L are at a much greater increased risk of hemorrhage. Abnormal or compromised platelet function or vascular disease in a thrombocytopenic animal can increase the risk of hemorrhage.

LABORATORY EVALUATION OF THROMBOCYTOPENIA

See Figure 77.1.

Complete Blood Count

The first indication that an animal is thrombocytopenic may come from a CBC. Likewise, a CBC should be the first test performed in any animal that is suspected of having a bleeding disorder. Generally EDTA-anticoagulated blood is acceptable for platelet counts for 5 hours after collection when kept at 20°C and 24 hours when kept at 4°C; however, certain platelet parameters are affected by anticoagulant, time or refrigeration.⁶⁴ Specific platelet parameters available on most automated CBCs include platelet count, mean platelet volume, and platelet distribution width. Additional

parameters available on some hematology analyzers may include plateletcrit, mean platelet component concentration, and platelet component concentration distribution width. Many of these platelet indices may provide helpful information about the underlying cause of thrombocytopenia; however, there is a paucity of studies in veterinary species, especially in those with thrombocytopenia. In addition to these automated parameters, microscopic examination of the blood smear for platelet morphology and size, and evidence of platelet clumping or adherence to leukocytes should be performed. Although manual methods are less accurate and precise compared to automated methods for platelet enumeration, they become necessary when platelet counts fall below the limit of sensitivity or linearity (usually 5,000–20,000 platelets/ μ L) of a hematology analyzer. Errors in manual platelet counts are less when performed by those who are experienced with the method and perform it regularly. If the platelet count is normal in an animal that has abnormal bleeding, petechiation, or ecchymosis, an abnormality in platelet function or a coagulopathy should be considered.

Platelet Parameters on Automated Hematology Analyzers

Mean platelet volume (MPV) estimates platelet size and is inversely proportional to platelet number in some species. Increases in MPV may be associated with response to thrombocytopenia, with some myeloproliferative diseases, and with hyperthyroidism.^{24,88} Decreases in MPV have been reported in some dogs that have early manifestations of immune-mediated thrombocytopenia.⁶⁷ Decreases in MPV are more often associated with bone marrow failure. Artifacts increases in MPV arise when platelets are exposed to EDTA for prolonged periods of time, cooled to room temperature (25°C), or refrigerated (4°C), or if there is delayed exposure to an anticoagulant. Minimal changes in MPV occur when blood is collected in an anticoagulant containing citrate and is kept at 37°C.^{35,64}

Platelet distribution width (PDW) is a value given by some automated particle counters and represents an index of variation in platelet size. PDW may be useful in laboratories that do not routinely perform smear evaluation. In humans, PDW was increased in patients with immune-mediated thrombocytopenia compared to patients with thrombocytopenia due to aplastic anemia; therefore PDW may be useful in differentiating between hyperdestructive thrombocytopenia (immune-mediated thrombocytopenia) and hypoproliferative thrombocytopenia due to aplastic anemia.⁴⁶

The plateletcrit (PCT or thrombocrit) is used to assess total circulating platelet mass and is the percentage of the blood volume that is composed of platelets. It is determined by certain analyzers or calculated using platelet count and MPV. Expected values are usually less than 1%. In dogs with experimentally-induced endotoxemia and thrombocytopenia, the platelet count and the PCT were significantly decreased, and the MPV and PDW were increased.¹⁰⁶ Because the PCT

represents total platelet mass, this parameter may be more representative of the functional potential and thrombopoietic stimulus than the platelet count.⁵³ PCT was found to be a better parameter than platelet count for assessing the platelet status in Cavalier King Charles Spaniels, a breed which can have a hereditary macrothrombocytopenia.¹⁰⁰

Mean platelet component concentration (MPC) estimates the average platelet density by two-angle light scatter and is used to assess platelet activation.¹⁵ MPC was found to be sensitive to the activation state of feline platelets.¹⁰⁸ MPC was decreased in several groups of horses including septic foals, foals with DIC and adult horses with inflammation, gastrointestinal obstruction, enteritis, ischemia, or thrombocytopenia, leading the authors to suggest that the parameter may be of value in detecting platelet activation in horses.⁸⁴ MPC is influenced by several factors including anticoagulant, sample age, storage temperature and time, so these should be considered when interpreting MPC results.^{26,92,101}

Platelet component concentration distribution width (PCDW) is a measure of the variability of platelet density, and values decrease with platelet activation and platelet shape change from discoid to spherical as platelets become activated. PCDW also decreases with increasing storage time and may be useful for documenting the platelet storage lesion.⁵⁵ Although studies in thrombocytopenic animals have not yet been done, PCDW may help elucidate potential causes of thrombocytopenia secondary to platelet activation.

Microscopic Evaluation of the Blood Smear

Microscopic examination of a blood film is necessary to evaluate platelet morphology and to ensure accuracy of automated platelet counts. Furthermore, decreased platelet counts should always be confirmed by microscopic examination of a blood smear, and if necessary, the platelet count should be repeated on a freshly drawn blood sample. It is also imperative that the venipuncture is carefully performed to prevent platelet activation resulting in artifactual changes on the CBC, making platelet parameters unreliable. Delayed time of exposure to anticoagulation, improper mixing, or improper collection can all contribute to falsely low platelet counts. EDTA-dependent pseudothrombocytopenia can be ruled out by restoration of normal platelet numbers and elimination of platelet clumps observed on blood smears when samples are drawn into citrate anticoagulant.

Evaluation of the blood smear should begin on low magnification to look for the presence of large platelet clumps. Often clumps are found on the feathered edge of the smear. If large clumps are found, this likely means that the number of platelets indicated by the hematology analyzer is falsely low. A general guideline for estimating platelet numbers on a blood smear is to determine the average number of platelets in 10 oil immersion fields using a 100× objective and multiply the average by 15×10^3 to obtain the estimated number of platelets per microliter.

Additional Diagnostic Tests

Once an animal is found to be thrombocytopenic, additional diagnostic testing is often necessary to elucidate the cause. Additional tests that should be considered include a coagulation panel, bone marrow analysis, serology or DNA analysis for infectious agents, and bacterial cultures. In thrombocytopenic horses, an agar gel immunodiffusion test (Coggins test) or an enzyme-linked immunosorbent assay (ELISA) to rule out equine infectious anemia is indicated.

Antiplatelet or antimegakaryocytic antibody assays, reticulated platelet analyses, and platelet function studies are additional tests that may be considered for the evaluation of platelet disorders. Antiplatelet antibody assays measure platelet-associated antibodies and may help to confirm an immune component but do not distinguish between primary and secondary immune-mediated thrombocytopenia. Reticulated platelets are young platelets recently released from bone marrow and contain increased RNA concentrations.^{4,19} In cases of peripheral platelet destruction, the percentage of reticulated platelets is increased in circulation. Thrombocytopenic patients who have compromised platelet production have decreased percentages of reticulated platelets in circulation.⁵⁰ The percentage of reticulated platelets was increased in two horses that had DIC and several ponies experimentally infected with equine infectious anemia virus.⁸⁰ In dogs, reticulated platelets may be a useful parameter for assessing platelet production.^{60,89} Many platelet function assays are only available at specialized or institutional laboratories and may not be readily available or practical.

Coagulation Panel

Performing a coagulation screen is suggested in any animal where a coagulopathy is suspected. A coagulation panel generally includes some combination of the following tests: activated partial thromboplastin time (APTT), one-stage prothrombin time (OSPT; also referred to as prothrombin time or PT), fibrinogen concentration, fibrinogen and fibrinogen-fibrin degradation products (FDP), antithrombin, and D-dimer concentration. Depending on the underlying pathology, animals may be thrombocytopenic without additional abnormalities on a coagulation panel or they may have abnormalities on a coagulation panel and not have thrombocytopenia. DIC secondary to a large variety of disorders and rodenticide toxicity are two conditions that can be seen in animals that are thrombocytopenic and have abnormal coagulation results.

Bone Marrow Evaluation

Bone marrow evaluation of megakaryocytes may be necessary to determine if thrombocytopenia is secondary to a platelet production defect or is caused by peripheral platelet destruction or consumption. The typical finding in cases of diminished or defective platelet production is the absence of or the decreased

numbers of megakaryocytes. Abnormal morphology and size may also be observed. In most cases of peripheral platelet destruction, megakaryocyte numbers are normal to increased. Often megakaryocytes are increased in size and ploidy as well. Both aspiration and core biopsies of bone marrow should be taken since both approaches offer certain advantages. Cellular morphology is superior with aspiration biopsies, whereas core biopsies are more useful for evaluation of overall megakaryocyte numbers, myelofibrosis, or necrosis. For proper and complete bone marrow interpretation, a blood sample should be taken for a CBC at the time of bone marrow sampling.

Bone marrow is collected by sterile technique, usually under sedation, but sometimes under general anesthesia. Sites for marrow collection can depend on personal preference and experience. The site is clipped and surgically prepared. A local anesthetic agent should be used in non-anesthetized animals. Use of a bone marrow needle with stylet (usually 15 gauge) is recommended. The needle with stylet is seeded into the bone and advanced a few more millimeters. The stylet is removed and a syringe (usually 10 or 20 mL) containing an anticoagulant (usually EDTA or citrate) is attached. If marrow is collected by this method, then the sample can be put into a Petri dish and grossly examined for particles. These appear as small flecks of whitish-gray material. Slides for cytology are made by picking these flecks up with a pipette and making squash preparations. Often, a bone marrow core biopsy can be taken when an aspirate is collected. After marrow has been aspirated, the needle is left in the bone and rotated 360° and advanced for another several millimeters and then pulled straight out. The stylet can then be used to push the core biopsy out of the needle so that it can be placed into formalin and processed.

LABORATORY CONFIRMATION OF CONSUMPTIVE THROMBOCYTOPENIAS

Thrombocytopenia and microangiopathic anemia with schistocytes are typical laboratory findings in DIC and TTP-HUS. Differentiation can often be made with the help of coagulation screening tests, including fibrinogen, FDP, D-dimer concentration, antithrombin levels, OSPT, and APTT. In DIC the classical laboratory findings are three or more of the following: thrombocytopenia, decreased plasma fibrinogen concentration, increased plasma FDP concentration, and prolonged APTT and OSPT. More recently, D-dimer testing has been introduced to many laboratories. D-dimers form from the degradation of cross-linked fibrin, whereas FDPs reflect either fibrinogen and/or fibrin breakdown. With DIC, elevations in D-dimers are often markedly increased, whereas low to moderately increased concentrations may be associated with a variety of disorders. One study in dogs reported that D-dimer concentration below 1000 ng/mL excluded most dogs that did not have DIC.⁹⁰ In foals where DIC was detected using clinicopathological criteria, D-dimer concentra-

tions were higher compared to non-DIC groups and controls.¹ In cats, D-dimer concentrations may not be a useful test for diagnosing DIC.^{7,91} Antithrombin is a molecule that binds thrombin to form a complex which is subsequently removed from circulation by hepatocytes. In DIC, antithrombin activity is usually decreased because of accelerated consumption. In contrast to DIC, coagulation tests (APTT, OSPT, fibrinogen concentration) are usually normal, FDP concentrations may be mildly increased, and D-dimer concentrations are increased in patients with TTP and HUS.^{43,62}

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Immune-Mediated Thrombocytopenia

MICHAEL A. SCOTT and L. ARI JUTKOWITZ

Primary IMT in Dogs

- Signalment
- History and Physical Exam
- Pathogenesis
- Diagnosis
 - IMT Assays
- Therapy
- Outcome

Primary IMT in Cats

- Signalment
- History and Physical Exam

Pathogenesis

- Diagnosis
- Treatment
- Outcome

Primary IMT in Horses

Secondary IMT

- Systemic Autoimmune Diseases
- Neoplasia
- Infectious Diseases
- Drugs

Alloimmune Thrombocytopenias

Acronyms and Abbreviations

ANA, antinuclear antibodies; CBC, complete blood count; D-MIFA, direct megakaryocyte immunofluorescence assay; EIA, equine infectious anemia; FeLV, feline leukemia virus; FIP, feline infectious peritonitis; FIV, feline immunodeficiency virus; IMHA, immune-mediated hemolytic anemia; IMT, immune-mediated thrombocytopenia; LE, lupus erythematosus; MLV, modified live virus; MPV, mean platelet volume; PDW, platelet distribution width; PF3, platelet factor 3; PSAIg, platelet surface-associated immunoglobulin; RBC, red blood cell; SLE, systemic lupus erythematosus.

PRIMARY IMT IN DOGS

Signalment

Dogs of any age, gender, or breed can suffer from primary IMT. The age of dogs with IMT has ranged from 3 months to 17 years, with reported mean or median ages of 4–8 years.^{25,31,42,49,52,56,57,69} In most studies, females have outnumbered males 2:1 to 3:1.^{25,30,31,33,42,52,69}

Some investigators have found no breed predisposition, while others have reported predispositions in cocker spaniels, toy poodles, miniature poodles, standard poodles, old English sheepdogs, and German shepherds.^{25,31,41,42,57,69}

History and Physical Exam

Most dogs with primary IMT are presented because of hemorrhage involving skin and mucosal surfaces.^{52,69} Hemorrhage may arise spontaneously or it may be a pathologic prolongation of bleeding following estrus, whelping, surgery, dentistry, grooming, or venipunc-

ture. Other nonspecific signs include lethargy, anorexia, stiffness, collapse, or weakness.^{4,30,69} Thrombocytopenia may also be discovered incidentally during routine health checks, preoperative testing, or medical evaluation of unrelated problems.^{52,57} Even dogs with platelet concentrations of less than 10,000/ μ L may be bright and alert with no clinical signs of disease (M.A. Scott, L.A. Jutkowitz, personal observations).

Physical examination of most affected dogs reveals petechiae, ecchymoses, epistaxis, gastrointestinal hemorrhage (melena, hematemesis, hematochezia), oral bleeding, vaginal bleeding, hemoptysis, and/or hematuria.^{31,33,52,69} Hemorrhages are especially common on the oral mucosae, ventral abdomen (Fig. 78.1), inner limbs, and pinnae, where they may be mistaken for a rash. Ocular lesions are also common and vary from mild conjunctival, scleral (Fig. 78.2), iridal, or retinal hemorrhage to hyphema, severe retinal hemorrhage, retinal detachment, and blindness.^{30,52,59} When hemorrhage is severe enough to cause anemia, other signs such as pallor, exercise intolerance, tachycardia, systolic ejection murmur, bounding pulses, and shock may



FIGURE 78.1 Abdominal petechiae and ecchymoses in a 7-year-old spayed German shepherd with primary immune-mediated thrombocytopenia.

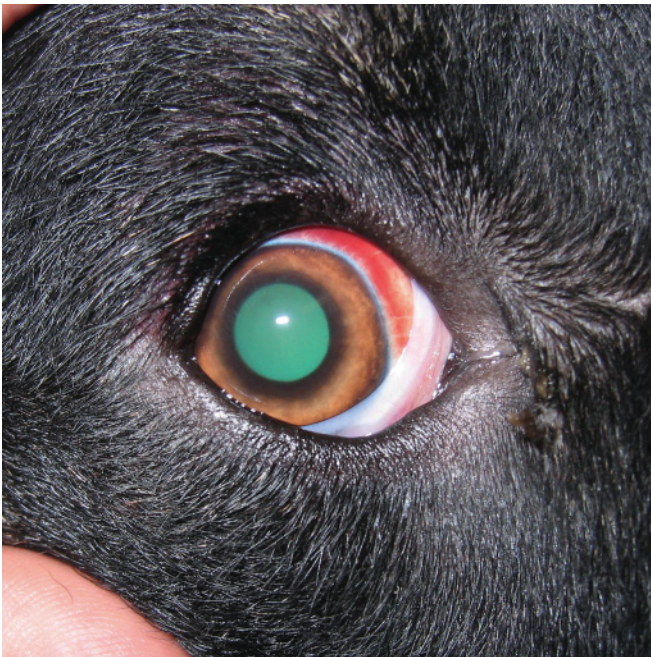


FIGURE 78.2 Scleral hemorrhage (present bilaterally) in a young dog with immune-mediated thrombocytopenia and 7,000 platelets/ μ L.

develop. Severe hemorrhage is most commonly attributed to gastrointestinal bleeding,⁴⁶ but it has also been reported secondary to epistaxis, hematuria, and vulvar bleeding.⁶⁹ Fever has been reported in many dogs with IMT, but it may be less common in dogs with primary IMT than in dogs with secondary IMT.^{31,33,52,69} Splenomegaly and lymphadenomegaly have been

reported. Intracranial and spinal cord hemorrhage are uncommon but life-threatening complications of IMT that should be suspected with decreasing levels of consciousness or acute onset of neurologic signs.

Pathogenesis

Primary IMT is an autoimmune disorder, unassociated with known predisposing conditions or multi-system autoimmune disease, in which the immune system causes thrombocytopenia through autoreactive mechanisms. It may be considered idiopathic to the extent that the root causes of autoreactivity are unknown. In human medicine, acute and chronic forms have been described. Human chronic IMT appears to be caused by varying degrees of accelerated platelet destruction and impaired platelet production mediated by humoral and cellular immune responses. T lymphocytes are reportedly resistant to apoptosis, suggesting that there is defective clearance of autoreactive T cells.⁴⁸ These autoreactive T cell clones appear to proliferate in response to platelet or megakaryocyte autoantigens and stimulate B cells to produce autoantibodies that mediate platelet destruction and impair megakaryocyte maturation and thrombopoiesis. Evidence also supports a role for cytotoxic T cells in platelet lysis and possibly in megakaryocyte damage. In acute childhood IMT, immune stimulation of an immature immune system may enable previously suppressed autoreactive B cells to escape thymic deletion because of cross-reactivity with other antigens (e.g. viral epitopes). As in chronic IMT, resultant autoantibodies may bind to platelets and megakaryocytes.

Early case reports describing idiopathic thrombocytopenia in dogs were likely reports of primary IMT.²³ The first laboratory evidence for this was positive results of the platelet factor 3 (PF3) assay in which globulin fractions from affected thrombocytopenic dogs accelerated coagulation, thus implicating the presence of antiplatelet antibodies. Later, direct assays for canine platelet surface-associated immunoglobulin (PSA Ig) supported the presence of immunoglobulins, usually IgG, on the platelets of dogs with clinical diagnoses of primary IMT.^{42,52,57} Acid eluates of platelets from some affected dogs were shown to contain immunoglobulins that immunoprecipitated the integrin $\alpha_{IIb}\beta_3$ from allogeneic platelets.⁴¹ Other canine IMT plasma has been shown to immunoprecipitate glycoprotein Ib/IX,⁵⁷ further documenting antibody binding to platelet glycoproteins.

Many human antiplatelet antibodies also bind to shared epitopes on megakaryocytes, and some appear to interfere with megakaryocyte maturation and thrombopoiesis despite normal to increased megakaryocyte numbers.¹³ Therefore, megakaryocytic hyperplasia should be interpreted only as increased megakaryopoiesis, not as evidence of increased thrombopoiesis. As expected, canine platelets and megakaryocytes have been documented to share some reactive sites, including epitopes on glycoprotein IIIa.¹⁷ Dogs with IMT or those made thrombocytopenic by injections of rabbit

anti-canine platelet antiserum have developed marked thrombocytopenia associated with megakaryocyte cytoplasmic vacuolation, foaminess, basophilia, and decreased granularity.³³ These microscopic changes may be indicative of immune-mediated megakaryocyte damage and impaired thrombopoiesis.

Rarely, autoantibodies to hematopoietic cytokines have been documented to induce IMT in people through impaired platelet production.²⁷ Similarly, injections of a recombinant human thrombopoietin product into dogs caused thrombocytopenia and megakaryocytic hypoplasia in association with increased titers of antibodies that were presumed to cross-react with canine thrombopoietin.¹⁶

Diagnosis

The diagnosis of primary IMT is one of exclusion, requiring clinical and laboratory evaluation (see Chapter 77) for all other causes of thrombocytopenia (Table 78.1).⁵¹ One must exclude non-immune causes of decreased platelet production, non-immune platelet consumption, abnormal platelet distribution, alloimmune IMT, and secondary IMT. Patient history should include exposure to drugs or toxicants, recent vaccinations, recent trips, recent contact with other dogs, previous transfusions, previous and current medical conditions, and tick exposure. Physical examination should include assessment of other causes of thrombocytopenia. Underlying infections may be suggested by lymphadenomegaly, ticks, arthritis, or fever. Neoplasia may be suggested by the presence of lymphadenomegaly, splenomegaly, other masses, or cachexia. Systemic immune-mediated disease may be suggested by polyarthritis or certain forms of dermatitis.

The extent and type of diagnostic testing varies with clinical information and geographic location, but typically includes: CBC, chemistry profile, and urinalysis; hemostasis profile for evidence of consumptive coagulation; abdominal and thoracic radiography and abdominal ultrasonography for organomegaly and occult neoplasia; and assessment for infectious agents by blood culture, serology, or molecular testing, as indicated. Bone marrow examination may be indicated, as well as tests for antinuclear antibodies (ANA), rheumatoid factor, lupus erythematosus (LE) cells, and red blood cell (RBC)- or neutrophil-associated immunoglobulin if systemic immune-mediated disease is suspected. Blood smears should be evaluated to confirm automated platelet concentrations and to exclude pseudothrombocytopenia. Pseudothrombocytopenia usually occurs when platelets are clumped because of platelet activation during or after venipuncture. Platelet clumps indicate the need for a new sample to be collected with a nontraumatic venipuncture. If anticoagulant-induced clumping is suspected, a new sample should be tested promptly after collection into a citrate tube. Pseudothrombocytopenia may also occur if many platelets are larger than the high threshold setting of automated hematology analyzers, in which case platelet

TABLE 78.1 Causes of Thrombocytopenia

Decreased platelet production
Acquired amegakaryocytic thrombocytopenia (may be immune-mediated)
Bone marrow replacement (hemic or metastatic neoplasia, myelofibrosis, osteopetrosis)
Hereditary disorders of hematopoiesis (e.g. β 1-tubulin gene mutation)
Infections (hemic cell infection, cytokine suppression, myelonecrosis; usually multifactorial)
Irradiation, whole body or extensive
Megakaryocytic leukemia
Myelonecrosis (infections, neoplasia, toxicants)
Myelosuppressive toxicants
Predictable (e.g. chemotherapeutics, estrogens in dogs, bracken fern in ruminants, tricothecene mycotoxins)
Idiosyncratic (e.g. phenylbutazone, meclofenamic acid, trimethoprim-sulfamethoxazole, griseofulvin)
Decreased platelet survival
Immune-mediated destruction
Primary (idiopathic, autoimmune)
Secondary
Drugs (may be multifactorial)
Infections (usually multifactorial)
Neoplasia (usually multifactorial)
Systemic autoimmune disease (e.g. lupus erythematosus)
Alloimmune
Neonatal alloimmune thrombocytopenia
Post-transfusion purpura
Non-immune consumption
Disseminated consumptive coagulation (envenomations, hepatic disease, infections, necrosis, neoplasia, overheating, pancreatitis, septicemia)
Hemorrhage, acute and widespread (e.g. anticoagulant rodenticides)
Localized intravascular coagulation (thrombosis, hemangiosarcoma)
Vasculitis/endocarditis (infectious vasculitides, hemolytic uremic syndrome)
Abnormal platelet distribution (splenic sequestration)
Hemodilution by massive infusion of platelet-poor fluids

concentrations should be measured using a hemocytometer. Clinically normal cavalier King Charles spaniels with platelet microtubule instability have many large platelets and thrombocytopenia,¹⁸ requiring careful microscopic assessment of the platelet appearance and concentration before exploring other reasons for thrombocytopenia in this breed. Similarly, many healthy greyhounds have platelet concentrations below typical canine reference intervals (e.g. near 100,000/ μ L).⁶¹

In primary IMT, platelet concentrations are usually lower than 50,000/ μ L,^{23,30,46,57} and frequently lower than 10,000/ μ L.^{52,57} Overall, platelet concentrations in dogs with IMT have been significantly lower than those of dogs with presumed non-immune thrombocytopenias.^{25,46,57} Most reports associate bleeding with platelet concentrations lower than 30,000/ μ L or even 10,000/ μ L,^{52,69} but bleeding may occur at higher concentrations.⁵² Inconsistent bleeding tendencies among dogs

with similar platelet concentrations may relate to variations in vascular stability, trauma, the rapidity with which thrombocytopenia develops, the age and metabolic activity of the circulating platelets, or the antigenic specificities of antiplatelet antibodies.

Decreased mean platelet volume (MPV) has been associated with immune-mediated platelet destruction.^{46,69} In one study, low MPV appeared to be a specific indicator of primary IMT in dogs.⁴⁶ However, MPV results for dogs with non-immune thrombocytopenias of similar severity were not reported. In another retrospective canine study, MPV and platelet distribution width (PDW) were not useful in differentiating causes of thrombocytopenia.⁶

Regenerative or nonregenerative anemias are common at presentation and are usually associated with blood loss.^{25,30,31,52,69} Leukogram abnormalities are inconsistent and not useful in diagnosing primary IMT,^{25,30,52} but they may suggest underlying or concurrent disease. Complete blood count abnormalities suggesting secondary IMT or non-immune thrombocytopenia include: bicytopenia, pancytopenia, leukemia, a severe inflammatory leukogram, granular lymphocytosis, marked hyperproteinemia, intracellular organisms (e.g. fungi, ehrlichia, distemper inclusions), extracellular parasites such as microfilaria, and RBC abnormalities (e.g. spherocytes, schistocytes, dacryocytes).

Bone marrow evaluation is not necessary in most dogs suspected of having primary IMT. Megakaryocyte findings are not specific for primary IMT, and non-megakaryocytic abnormalities are uncommon in dogs for which primary IMT is the major diagnostic consideration.⁴⁵ Bone marrow assessment may be indicated initially to detect other causes of thrombocytopenia or after treatment in those dogs that do not respond well to routine therapy. Indications for bone marrow evaluation include suspicion of leukemia, multiple myeloma, or other hemic neoplasia. Other causes of production failure that may be identified include myelofibrosis, bone marrow necrosis, metastatic cancer, and granulomatous infections. If bone marrow hypoplasia or aplasia is suspected, a core biopsy should be done in addition to an aspirate. Thrombocytopenia should not be considered a contraindication to bone marrow sampling in thrombocytopenic dogs,⁶⁹ although prolonged pressure may be required to achieve hemostasis.

Estimation of marrow megakaryocyte numbers is subjective and dependent on sample quality. Reliable estimates should be attainable with either representative core samples or aspirate samples containing many marrow particles. However, megakaryocyte numbers can be underestimated in aspirate samples with few or no particles because megakaryocytes tend to be associated with particles. Compared to aspirate smears, core sections allow better differentiation of megakaryocyte numbers. In primary IMT, megakaryocytes are usually present in normal or increased numbers (Fig. 78.3).^{33,52,69} However, rare dogs have absent or markedly reduced numbers of megakaryocytes.^{9,33,39,69}

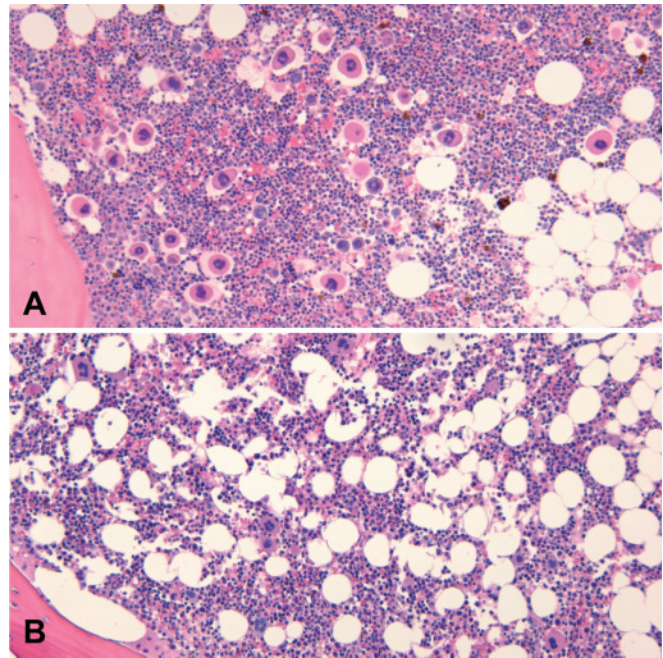


FIGURE 78.3 Bone marrow core biopsy samples.

(A) Megakaryocytic hyperplasia and erythroid hyperplasia in an 8-year-old castrated mixed breed dog with primary immune-mediated thrombocytopenia, a platelet concentration of 4,000/ μ L, and secondary hemorrhage. (B) In contrast, normal megakaryocyte density in an 8-year old castrated boxer with unexplained hypercalcemia. Megakaryocytes are large cells with multilobulated nuclei and eosinophilic cytoplasm. H&E stain.

IMT Assays

The diagnosis of primary IMT remains a diagnosis of exclusion because assays have insufficient diagnostic sensitivity and specificity for routine clinical utility.⁵¹ Early assays, such as the PF3 test, were insensitive and nonspecific indirect functional assays that cannot be recommended.^{12,31,33,69} Current assays have more analytical sensitivity and specificity for antibodies to platelets or megakaryocytes. The presence of increased PSAIg or other positive test results is evidence of an immune component to the thrombocytopenia. However, positive results do not differentiate primary IMT from secondary IMT, and PSAIg has been increased in human patients with presumed non-immune thrombocytopenias.⁵¹ Although patients with negative results need to be evaluated further for non-immune causes of thrombocytopenia, negative results may occur in primary IMT. Therefore, all causes of thrombocytopenia must be considered despite assay results.

Most assays developed to diagnose IMT in dogs have used labeled antiglobulin reagents to detect platelet- or megakaryocyte-reactive immunoglobulins, and none are glycoprotein specific. These assays include the megakaryocyte immunofluorescence assays D-MIFA,^{31,33,37} enzyme-linked immunosorbent assays,^{12,42,44} platelet immunoradiometric assays,⁵⁷ and microscopic and flow cytometric platelet immunofluorescence assays.^{37,52}

Flow cytometric assays are currently used most frequently. Assays have been designed either as direct assays that assess for antibodies on patient platelets or megakaryocytes, or as indirect assays that assess for antibodies in patient plasma or serum that bind to platelets or megakaryocytes from healthy individuals (see Chapter 140).

Direct assays are generally superior to indirect assays in assessing primary IMT. Indirect assays cannot differentiate plasma or serum autoantibodies from confounding circulating immune complexes, aggregates of IgG formed in heated or stored sera, or acquired and naturally-occurring alloantibodies to common or platelet-specific antigens. However, transport and storage of samples may profoundly and unpredictably affect the results of some direct assays.⁵⁷ D-MIFAs are little affected by sample transport, but D-MIFA positivity may not mirror PSAIg positivity.

Therapy

When treating dogs with IMT, aspirin and other antiplatelet drugs should be avoided. Medications that may cause or contribute to thrombocytopenia should be discontinued whenever possible. Trauma (including intramuscular injections) should be minimized, jugular venipuncture avoided, and concurrent problems treated appropriately. Other therapy should be aimed at raising platelet concentrations enough to protect against clinical hemorrhage, though some dogs may do well despite low platelet concentrations and no medical management.³¹ Platelet concentrations within the reference interval are the preferred target and may be achieved in the majority of patients.⁵² However, some patients may be refractory to therapy or intolerant of treatment side effects. In such patients, reaching platelet concentrations of 50,000–100,000/ μ L is probably adequate in the absence of platelet dysfunction.⁵²

Immunosuppressive doses of corticosteroid are the mainstay of therapy in dogs, reportedly stabilizing vascular endothelium, decreasing macrophage phagocytosis of opsonized platelets, increasing platelet production, and diminishing antibody production.⁵² Prednisone or prednisolone is generally started at a dose of 1–2 mg/kg every 12 hours; an equivalent dose of dexamethasone may be calculated by dividing the prednisone dose by seven.⁷ There is currently no evidence to suggest that the higher end of the immunosuppressive range is more effective than the lower end, or that dexamethasone is more effective than prednisone in dogs. In many dogs, hemorrhage may resolve prior to increases in platelet concentration.^{30,69} The glucocorticoid dose is gradually tapered over several months until being withdrawn or maintained at the minimal effective dose as directed by platelet concentrations. Side effects or inadequate response justify other therapeutic approaches.

Numerous adjunct immunosuppressive agents have been used in dogs with IMT. Decisions regarding the selection of these drugs are frequently based upon clinical bias, as randomized, controlled, clinical trials in

dogs with IMT are lacking. However, beneficial effects of adjunct agents cannot be excluded, and combination therapy with reduced doses of individual drugs may reduce unwanted side effects. Azathioprine, cyclophosphamide, danazol, cyclosporine, mycophenolate, vincristine, and human gamma globulin have all been used.^{4,30,31,52,56,69}

In one prospective, controlled, but non-randomized study, combined therapy with prednisone and vincristine (0.02 mg/kg once intravenously) was associated with a more rapid increase in platelet concentration and shortened duration of hospitalization compared to treatment with prednisone alone.⁵⁶

In dogs, splenectomy has had variable results.^{30,69} It remains to be seen if response to splenectomy can be predicted. Hemotropic mycoplasmosis is a recognized complication of splenectomy and immunosuppression in dogs but long-term studies of this problem in clinical cases of IMT are lacking.²

There is little evidence to support the prophylactic administration of platelet products to dogs with IMT. Studies to date addressing this issue failed to find a benefit in dogs that received platelet-rich plasma or fresh whole blood. Transfused platelets are rapidly consumed or destroyed by circulating antiplatelet antibodies. However, significant transient increments may occur, and, as in human patients, they justify platelet transfusions when dogs with IMT require surgery or when there is severe, life-threatening hemorrhage. Multiple units of platelet concentrates are usually required for an appreciable effect. Additionally, an estimated 30% of dogs with IMT require packed RBC transfusions to address acute blood loss.^{52,56}

Rituximab, a monoclonal antibody that targets the CD20 antigen on B cells, has been used successfully in human patients to treat lymphoma as well as IMT.⁵³ The goal of this and other antibodies evaluated for therapy of human IMT is to disrupt the immune dysregulation driving autoantibody production. However, the monoclonal antibody to human CD20 does not appear to bind canine B cells.²⁹ With increasing evidence that platelet production may be impaired in IMT, treatments that promote megakaryopoiesis may also be helpful. Romiplostim (Amgen Inc., Thousand Oaks, CA), a thrombopoietin receptor ligand, has recently received approval for the treatment of chronic IMT in people. In a phase 3 clinical trial, 87% of patients given Romiplostim were able to discontinue or decrease concurrent therapy, compared to 38% in the placebo group.³⁸ During pre-clinical development, Romiplostim was studied in mice, rats, rabbits, dogs, and monkeys and was reported to increase platelet concentrations in the majority of species investigated.⁶⁸

Outcome

The prognosis for dogs with primary IMT is generally favorable, with initial response rates of 80–95%.^{4,30,52,56} Most dogs achieve hemostatic platelet concentrations (>50,000/ μ L) within 5–7 days, but recovery times of up to 35 days have been reported.^{4,30,52,56} Dogs that do not

respond to initial therapy may still have a favorable outcome given more time or alternative strategies, such as splenectomy. Reported mortality rates have ranged from 10% to 43%, with the lower rate⁵² more in line with the authors' experience. Both acute and chronic forms of the disease are seen in dogs, perhaps analogous to the human conditions. Over 50% of dogs with IMT experience a single, acute bout of thrombocytopenia followed by recovery.^{30,52,69} However, a substantial percentage of dogs respond initially but relapse and require re-treatment or chronic therapy with low dose steroids or other immunosuppressive agents.^{30,52,69} There is currently no way to predict which outcome a particular dog will have.

PRIMARY IMT IN CATS

Signalment

Primary IMT appears to be a rare condition in cats, with only a few probable cases reported.^{5,22,32,34,36,63} The ages of affected cats ranged from 18 months to 12 years with a median age of 6 years. A gender bias is not apparent. Affected cats have been domestic shorthairs and single cats of the following breeds: Abyssinian, Somali, and British shorthair.

History and Physical Exam

Presenting complaints and physical exam findings include epistaxis, petechiae, ecchymoses, hematochezia, hematuria, and hemoptysis.^{5,22,32,34,36,63} Decreased appetite and weight loss have also been present.⁵

Pathogenesis

An immunologic pathogenesis for reported cases of feline idiopathic thrombocytopenia has been supported by exclusion of other identifiable disorders, response to immunosuppressive therapy, or increased PSAIg.³⁶ The presence of megakaryocytic hyperplasia in most reported cases supports a decrease in platelet survival with responsive megakaryopoiesis.

Diagnosis

The diagnosis of IMT in cats is one of exclusion. Most affected cats have severe thrombocytopenia (<15,000/ μ L) and mucocutaneous hemorrhage. Low automated platelet concentrations should be confirmed by evaluation of a blood film or by determining the platelet concentration with a hemocytometer. Pseudothrombocytopenia is common in cats, secondary to platelet clumping and/or large platelets that may not be counted as platelets by automated analyzers. Once true thrombocytopenia is confirmed, all causes of thrombocytopenia should be considered, including decreased platelet production, increased platelet consumption, abnormal platelet distribution, and accelerated platelet destruction by immune or non-immune mechanisms (Table

78.1).³² Acquisition of historical information, physical examination, and diagnostic testing should generally mirror those for the thrombocytopenic dog (see page 588). In the cat, additional consideration should be given to certain drug exposures (e.g. methimazole)⁵⁰ and to infection with FIV or FeLV.³⁶ In one retrospective study, thrombocytopenia was associated most commonly with viral infections, especially by FeLV, and with neoplasia, particularly hemic in origin.³² Therefore, bone marrow examination is indicated. Regenerative and nonregenerative anemias have been reported in cats with primary IMT, and they are not unexpected with blood loss.⁵ However, anemia may occur with concurrent IMHA (i.e. Evans' syndrome)³⁶ or other illness resulting in anemia and a non-immune or secondary immune-mediated thrombocytopenia.

Relatively little work has been done to develop and validate feline assays for PSAIg,^{34,36,63} and, as in dogs and people, these tests will likely play a limited role in the clinical diagnosis of primary IMT. Antiplatelet antibodies may support an immunologic component to the thrombocytopenia if the assays are reliable, but current assays cannot differentiate primary IMT from secondary IMT. Increased PSAIg has been reported in cats with a wide array of disorders including fat necrosis, feline infectious peritonitis (FIP), FeLV infection, FIV infection, lymphoma, leukemia, hepatitis, pyelonephritis, and hyperthyroidism.³⁶

When no underlying condition is found in a severely thrombocytopenic cat with megakaryocytic hyperplasia and a thorough diagnostic work-up, idiopathic IMT is a reasonable working diagnosis, particularly if a reliable assay indicates the presence of increased PSAIg. Positive response to immunosuppressive therapy may lend further support to the conclusion.

Treatment

Therapy for cats with idiopathic IMT is similar to that of dogs. Prednisolone may be initiated at a dose of 2mg/kg orally twice daily, or an equivalent dose of dexamethasone (approximately 0.3mg/kg) may be given intravenously at the same frequency. Prednisone may be inferior to prednisolone in cats as it appears to have marked variability in bioavailability and activity in this species.²⁴ Cyclosporine, azathioprine, and vincristine have been used in cats with IMT, with variable success.^{5,22,63} Intravenous immunoglobulins may be used in cats with refractory disease.⁷⁰

Outcome

The prognosis for cats with primary IMT is similar to that for dogs, with over 80% of the reported cats surviving 15 months to greater than 5 years from the time of diagnosis.^{5,22,32,34,36,63} Median recovery time to hemostatic platelet concentrations (>50,000/ μ L) has been 7 days, ranging from three days to 144.^{5,22,36,63} All reported cats with IMT required long-term therapy, and some never achieved normal platelet concentrations.^{5,22,36,63} Relapses were reported in cats weaned from therapy.

PRIMARY IMT IN HORSES

Immune-mediated thrombocytopenia is uncommon in horses, but it may occur as idiopathic thrombocytopenia (likely primary),^{11,28,43} neonatal alloimmune thrombocytopenia, or in association with infections, drugs, neoplasia, or IMHA.

As in other species, the diagnosis of primary IMT is based on exclusion of other causes of thrombocytopenia. Thrombocytopenia may be suspected because of the presence of mucocutaneous hemorrhage (e.g. petechiae, epistaxis) and prolongation of bleeding from sites of trauma, including venipuncture. Complete blood count, chemistry, urinalysis, and hemostasis profiles should be performed to document thrombocytopenia and screen for underlying disorders. Thrombocytopenia is typically severe and associated with hemorrhage, but it should be confirmed with microscopic examination of a blood film to exclude pseudothrombocytopenia. Equine platelets tend to be pale staining and together with the relatively low numbers of platelets in horses, when compared to other species, blood film evaluation may yield a false impression of thrombocytopenia to the untrained eye. History and physical examination should be thoroughly assessed for known causes of thrombocytopenia (see Table 78.1). Bone marrow assessment may be useful to detect underlying diseases. Molecular or serologic diagnostics for infections with equine infectious anemia virus and *Anaplasma phagocytophilum* may also be indicated. A platelet antibody assay may be used to provide evidence of PSAIg.⁴³

Most horses with idiopathic IMT respond favorably to corticosteroids, but azathioprine has been added in refractory cases with apparent benefit.^{28,43} Dexamethasone may be instituted intravenously at 0.1–0.2 mg/kg every 24 hours. It may be continued with tapering doses over several weeks, or therapy may be switched to tapering doses of oral prednisolone. Prednisone should not be used because it has limited absorption in horses.⁴⁹ Some horses experience only a single episode of thrombocytopenia while others may have recurrent bouts. Platelet-rich plasma may be used for life-threatening bleeding.

SECONDARY IMT

Secondary IMT refers to IMT that is part of a more widespread autoimmune disease or that is associated with known predisposing neoplastic, infectious, or drug-induced conditions. Classification as secondary IMT is justified when there is a clear association between thrombocytopenia and the underlying condition, and when there is strong evidence that immune mechanisms are responsible for the thrombocytopenia. Increased PSAIg in a patient with a particular disorder supports an immunologic component to the thrombocytopenia, but it would be insufficient evidence to conclude that the underlying disorder should be considered a definite cause of secondary IMT.

In patients with secondary IMT, PSAIg may be bound specifically to platelet autoantigens as antiplatelet autoantibodies (e.g. systemic lupus erythematosus) or it may be bound to adsorbed non-autoantigens associated with infectious agents, drugs, or neoplasia (Fig. 78.4). PSAIg may also be in the form of immune complexes bound to platelets by: (1) complement-mediated immune adherence; (2) Fc receptors for IgG in species with platelet Fc γ receptors; or (3) nonspecific interactions. Circulating immune complexes may arise from infectious diseases, vaccinations, drugs, neoplasia, or systemic autoimmune diseases. Therefore, secondary IMT may or may not be autoimmune. In veterinary medicine, the type of interaction between antibody and platelet surface is typically unknown for any particular patient with a positive platelet antibody result.

Systemic Autoimmune Diseases

Primary IMT is but one clinical presentation in a spectrum of somewhat indistinct autoimmune disorders with different antibody specificities. Systemic lupus erythematosus (SLE) associated with presumed IMT has been reported in dogs, cats, and horses (see Chapter 54). An immune-mediated pathogenesis of the thrombocytopenia in dogs with SLE has been supported by PSAIg positivity and positive results with D-MIFAs or indirect assays. Thrombocytopenia may accompany IMHA because of consumption or immune-mediated destruction (Evans' syndrome) (see Chapters 33 and 34).

Immune-mediated thrombocytopenia has been suspected in association with several other targets of the immune system. Positive indirect results for platelet-bindable immunoglobulin have been reported in a dog with steroid responsive idiopathic neutropenia and thrombocytopenia, and in a dog with thrombocytopenia and red cell aplasia.³⁷ Similarly, a syndrome of concurrent neutropenia, splenomegaly, and severe thrombocytopenia has been described in giant schnauzers.⁶⁵ IMT has been clinically diagnosed or suspected in dogs with rheumatoid arthritis and pemphigus, and thrombocytopenia has accompanied juvenile-onset polyarthritis syndrome in Akitas.^{21,25} Although possible IMT was reported in association with canine inflammatory bowel disease, thrombocytopenia was severe in only one dog and moderate in one dog.⁵⁵

Neoplasia

Thrombocytopenia frequently occurs in association with neoplasia, and although its pathogenesis is multifactorial, immune-mediated platelet destruction may contribute. This would explain the documented shortened platelet survivals without concurrent decreases in fibrinogen survivals in dogs with multicentric or metastatic neoplasms.⁴⁷ Lymphoma and other hemic and non-hemic tumors have been associated with IMT in dogs, and this is supported by results of D-MIFAs and indirect assays for platelet-bindable immunoglobulin.^{31,35,37} IMT also has been diagnosed in horses with lymphoma.

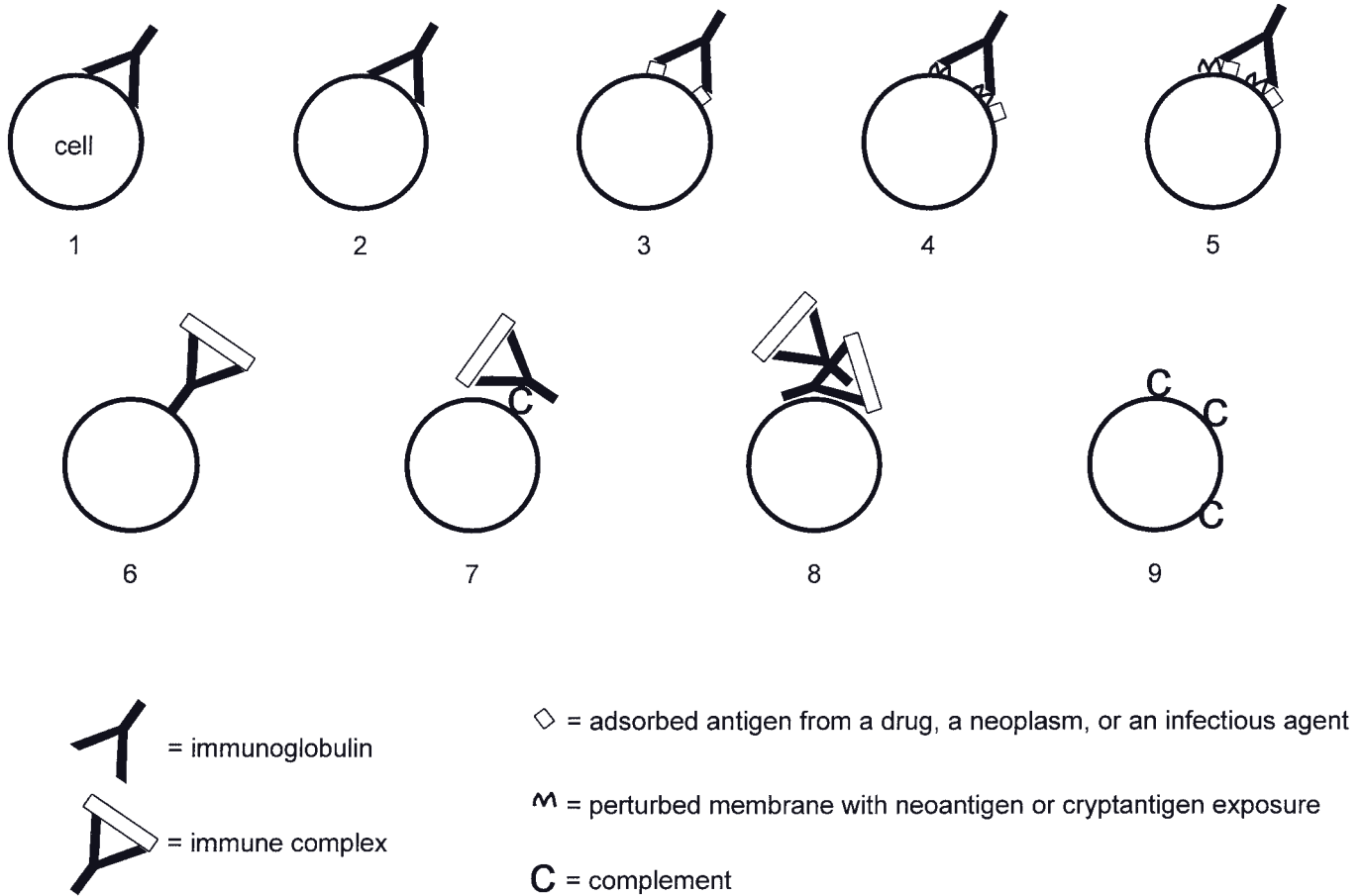


FIGURE 78.4 Schematic diagram of mechanisms by which immunoglobulins and complement may bind to and opsonize platelets or other hemic cells. Note that platelet surface-associated immunoglobulin (PSA Ig) is not necessarily caused by autoantibodies. 1. Autoantibody binding to specific autoantigens is Fab mediated; this occurs in primary immune-mediated thrombocytopenia (IMT). 2. Alloantibody binding is also Fab mediated, but antibodies bind to specific cell alloantigens; this occurs in transfusion reactions and neonatal alloimmune thrombocytopenia. 3–5. In secondary IMT, Fab-mediated binding may occur to adsorbed antigens from infectious agents, drugs, or neoplasms (3), to surface sites perturbed by other molecules (4), or to a combination of adsorbed antigen and perturbed membrane (5). 6. In secondary IMT, PSA Ig may also be bound as immune complexes via Fc receptors in some species (e.g. primates). 7. Immunoglobulin may bind via complement-mediated immune adherence in species with platelet complement receptors. 8. With in vitro indirect assays of serum or plasma, increased PSA Ig may be a storage artifact resulting from nonspecific binding of immunoglobulins that have aggregated during freezing. 9. Complement deposition may occur as an innocent bystander phenomenon, with cold-reacting antibodies that dissociate from the cells before testing, or along with any complement-activating PSA Ig.

Infectious Diseases

Thrombocytopenia is commonly associated with infections caused by viruses, bacteria (especially the rickettsials), protozoa, fungi, and nematodes.^{6,25,32,58} As with neoplasia, the pathogenesis of these infectious thrombocytopenias may be complicated, involving various combinations of suppressed platelet production, altered platelet distribution, increased consumption, or immune-mediated and non-immune platelet destruction.

Rickettsial diseases are common infectious causes of thrombocytopenia in endemic areas, but the pathogenesis of the thrombocytopenias are poorly understood. Immune-mediated platelet destruction is thought to contribute to the thrombocytopenia of acute ehrlichiosis caused by *Ehrlichia canis*,⁶⁷ and positive Coombs' tests

have suggested that immune-mediated disease may not be restricted to platelets.⁶⁶ PSA Ig or serum platelet-bindable immunoglobulins have been increased in some naturally and experimentally infected dogs.^{41,67} Immune mechanisms may contribute to thrombocytopenias in other rickettsial infections, including infection with *Anaplasma phagocytophilum*³ and *Rickettsia rickettsii*.²⁶

Cats with FIV and FeLV infections may develop thrombocytopenias with immune components (see Chapters 55 and 62). Immune-mediated platelet destruction appears to contribute to the thrombocytopenia associated with equine infectious anemia (EIA), but thrombocytopenia in EIA is likely multifactorial and caused partly due to impaired platelet production.¹⁵ Puppies experimentally infected with canine distemper virus develop marked thrombocytopenia and increased PSA Ig.¹ A complement-independent, immune

complex-induced destruction of platelets appeared to contribute to thrombocytopenia. Dogs⁶⁰ and other species²⁰ vaccinated with modified live virus (MLV) distemper vaccines may develop mild to occasionally marked transient decreases in platelet concentrations. While clinical purpura has reportedly followed 1–21 days after routine clinical MLV vaccinations,²⁰ further studies of these clinical observations have not been published.

Immune-mediated platelet destruction likely contributes to the thrombocytopenia occurring commonly in dogs with histoplasmosis, babesiosis, and leishmaniasis, and it may play a role in thrombocytopenic dogs with other fungal or nematode infections.^{8,14,42,62} Serum from dogs with leishmaniasis contains platelet-bindable IgM and sometimes IgG, suggesting a role for immune-mediated platelet clearance, but the nature of the platelet-immunoglobulin interaction is not known and may involve immune complexes and immune adherence.⁶⁴

Drugs

Drugs produce thrombocytopenia by many mechanisms, including immune-mediated platelet destruction by drug-induced antibodies that may be detectable by indirect assays of serum or plasma (see Chapters 14–16). Drug-induced antibodies may be drug-dependent, requiring the presence of the drug or its metabolite for platelet binding, or they may occasionally be drug-independent, behaving like autoantibodies that bind in the absence of drug. Some drug-dependent antibodies bind to cells in the form of drug-antibody complexes. Others bind directly to either membrane cryptantigen exposed by the presence of the drug or to drug-protein neoantigens on the cell surface. Drug-induced IMT has been suspected with several drugs, most notably sulfonamides in dogs,^{40,42} methimazole in cats,⁵⁰ and penicillin or trimethoprim-sulfadoxine in horses.⁴³

ALLOIMMUNE THROMBOCYTOPENIAS

Although alloimmune thrombocytopenias can be classified as types of secondary IMT, they are considered separately here because of their distinct pathogenesis. They result from the production of alloantibodies that target platelet alloantigens and cause platelet destruction, primarily by phagocytosis of the opsonized platelets. Exposure to foreign platelet alloantigens may occur with pregnancy or blood transfusion, causing neonatal alloimmune thrombocytopenia or post-transfusion purpura, respectively.

Neonatal alloimmune thrombocytopenia occurs when maternal antibodies to paternal epitopes on neonatal platelets are passively transferred through the placenta or colostrum. These antibodies circulate in the blood where they bind to neonatal platelets and lead to their premature destruction. The disorder is self-limiting because of the inherent lifespan and amounts

of transferred immunoglobulins. Neonatal alloimmune thrombocytopenia is uncommon, but has been described in pigs, horses, and possibly mules.^{10,19,54} The diagnosis of neonatal alloimmune thrombocytopenia is mostly one of exclusion, bacterial or viral infections being the most common differential. Where available, antibody assays may be applied to support a diagnosis of alloimmune thrombocytopenia by documenting the presence of PSAIg in the neonate, the absence of maternal PSAIg, and the presence of maternal antibody reactivity with paternal and neonatal, but not maternal, platelets. Specific therapy may not be required for this self-limiting condition, but glucocorticoids may be useful in severely affected newborns. Platelet transfusions are indicated if hemorrhage is severe or if central nervous system hemorrhage is suspected. Compatible platelets can be obtained from the dam, but they should be washed to remove plasma antibodies. Future offspring from the same mating pair are at risk of developing the same disease.

Post-transfusion purpura is characterized by severe thrombocytopenia developing several days after transfusion of a patient previously sensitized to a platelet alloantigen by pregnancy or transfusion. Re-exposure to the platelet alloantigen induces a high titer of antibodies targeting the transfused platelets. Severe thrombocytopenia occurs because the patient's own platelets are also destroyed, despite being negative for the target alloantigen when tested after recovery. Proposed mechanisms include epitope spreading, the development of an oligoclonal immune response that targets non-polymorphic autoepitopes in addition to the inciting alloepitope. Development of thrombocytopenia within 1–2 weeks of a blood transfusion should prompt consideration of this clinical entity in veterinary species (see Chapter 100).⁴²

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Non-Immune-Mediated Thrombocytopenia

JENNIFER S. THOMAS

Platelets and Normal Hemostasis

Platelet Function

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Acronyms and Abbreviations

APTT, activated partial thromboplastin time; AT, antithrombin; CBC, complete blood count; CDV, canine distemper virus; DIC, disseminated intravascular coagulation; EDTA, ethylenediaminetetraacetic acid; FDP, fibrin/fibrinogen degradation product; GPIIb-IIIa, glycoprotein IIb-IIIa; HUS, hemolytic uremic syndrome; IMT, immune mediated thrombocytopenia; MPC, mean platelet component; MPS, mononuclear phagocytic system; MPV, mean platelet volume; PCR, polymerase chain reaction; PSAIg, platelet surface associated immunoglobulin; PT, prothrombin time; TPO, thrombopoietin; TTP, thrombocytopenic thrombotic purpura; VWF, von Willebrand factor.

Thrombocytopenia is generally an acquired pathologic process that accompanies an underlying disease or disorder and is a common finding in many species.^{22,27,64} Uncomplicated, mild to moderate thrombocytopenia is usually subclinical and affected animals are either asymptomatic or have clinical signs (e.g. fever, lethargy, anorexia) that are related to the underlying condition. Thrombocytopenia is often first recognized when a decreased platelet concentration is identified following review of a blood smear or a complete blood count (CBC). Spontaneous bleeding disorders due to uncomplicated thrombocytopenia are rare when platelet concentrations are greater than 20,000–30,000/ μL .^{2,27}

Animals with severe thrombocytopenia or thrombocytopenia complicated by a coagulopathy, vascular disorder, or functional platelet defect often present with clinical findings typical of a defect in primary hemostasis. Findings may include petechiae or ecchymoses (often on the ventral abdomen, inner thighs, or mucous

membranes), epistaxis, gastrointestinal bleeding, gingival bleeding, hematuria, vaginal bleeding, or ocular hemorrhage. Excess hemorrhage following trauma, surgery, and venipuncture is sometimes noted.^{56,66}

PLATELETS AND NORMAL HEMOSTASIS

Platelet Function

Platelets play a central role in thrombus formation and maintenance of vascular integrity. Initially, platelets are recruited and help to form the primary hemostatic plug which can repair small lesions in the vascular wall. Subsequently, platelets play a role in fibrin production and formation of the more stable secondary hemostatic plug.⁶⁶

When a blood vessel is injured, platelets initially adhere to the defect in a von Willebrand factor (VWF)-dependent manner, particularly under high shear con-

ditions. Following adhesion, platelets become activated and expose fibrinogen binding sites on membrane integrin $\alpha_{IIb}\beta_3$, also known as glycoprotein IIb-IIIa (GPIIb-IIIa). Adhesive proteins, such as fibrinogen and VWF, bind to $\alpha_{IIb}\beta_3$ on adjacent platelets and form a bridge to support platelet aggregation. Activated platelets release a variety of agonists that activate other platelets (e.g. thromboxane, adenosine diphosphate), promote vessel healing (e.g. platelet derived growth factor), or play a role in coagulation (e.g. fibrinogen, factor V). Activated platelets shift negatively charged phospholipids (e.g. phosphatidylserine) from the inner membrane surface to the outer membrane where they provide binding sites for coagulation enzymes and cofactors.^{28,38} Finally, platelets facilitate wound closure by undergoing contractile processes that lead to clot retraction.⁶⁶

Platelet Kinetics

Platelet concentration in the blood reflects a balance between platelet production and platelet consumption, destruction or redistribution to the vasculature of organs. In most healthy animals, platelet lifespans are approximately 5–10 days.⁶⁶ Platelets are continually consumed during repair of small vascular defects. Aged platelets are removed by macrophages. The spleen appears to play a major role in determining platelet survival. Platelet lifespans are significantly longer in splenectomized dogs when compared to healthy dogs with spleens.¹¹

Studies in people have shown that 10^{11} platelets are produced every day and platelet population turnover occurs every 8–9 days. Platelets are produced by cytoplasmic fragmentation of megakaryocytes, with each megakaryocyte producing thousands of platelets.¹⁴ In response to increased demand, platelet production can increase 20 fold or more.³⁰ Platelet production is affected by a number of growth factors (see Chapter 9); however, thrombopoietin (TPO) plays the primary role in regulating megakaryopoiesis and thrombopoiesis.³⁰ Thrombopoietin is primarily produced by hepatocytes, stromal cells in the bone marrow, and renal epithelial cells. In healthy animals, TPO is constitutively produced and the blood concentration is generally inversely related to platelet mass.¹⁴ Thrombopoietin is bound to receptors on platelets and megakaryocytes. When circulating platelet mass is decreased, the concentration of unbound TPO available to stimulate megakaryopoiesis and thrombopoiesis is increased. Production of TPO is increased in some disease states. In inflammation, interleukin 6 has been shown to induce TPO production by the liver.¹⁴

DIAGNOSTIC ASSAYS FOR THROMBOCYTOPENIA

The laboratory evaluation of thrombocytopenia is covered in greater depth in Chapter 77. A list of common diagnostic assays is found in Table 79.1. When throm-

TABLE 79.1 Diagnostic Tests for Thrombocytopenia

Assay	Comments ^a
Complete blood count	
Platelet concentration	Automated or manual Confirm and track thrombocytopenia
Mean platelet volume	Increase suggests enhanced thrombopoiesis
Mean platelet component	Decrease suggests platelet activation
Blood smear examination	Detect platelet clumps Estimate platelet concentration Detect organisms or neoplastic cells Detect large platelets – suggests increased production Evaluate for inflammatory leukogram
Hemostasis profile	Evaluate for DIC
Bone marrow examination	Assess megakaryocyte density and morphology Detect organisms, neoplastic cells, necrosis, inflammation, myelofibrosis
Serology	Detect exposure to infectious agents
PCR	Detect infectious agents
Flow cytometry	Detect PSAIg Measure reticulated platelets to assess platelet production Detect activation markers

^aDIC, disseminated intravascular coagulation; PSAIg, platelet surface-associated immunoglobulin.

bocytopenia is detected by an automated hematology analyzer, it is important to first examine a blood smear to check for platelet clumps, estimate platelet concentration to confirm the accuracy of an automated platelet count, and evaluate platelet morphology. If clumps are present then the automated platelet concentration is of minimal value. Collection of a fresh blood sample without clumping would be required to determine an accurate platelet concentration.

Once thrombocytopenia is confirmed, additional qualitative and quantitative data are available from a routine CBC and provide useful diagnostic information. Identification of increased numbers of large platelets, also known as megaplatelets, on a blood smear suggests increased thrombopoiesis and may be associated with an increased mean platelet volume (MPV).⁶⁶ Increased MPV in an animal with thrombocytopenia suggests adequate to increased platelet production in the bone marrow.^{29,69} Decreased mean platelet component (MPC) suggests *in vivo* platelet activation.^{48,62}

Additional diagnostic tests are often required to determine the underlying cause of the thrombocytopenia. The decision of which tests to perform depends on clinical presentation and results of other diagnostic findings. A complete hemostasis profile (prothrombin time [PT], activated partial thromboplastin time [APTT], fibrinogen concentration, fibrin degradation products [FDPs], D-dimer, and antithrombin [AT] activity) is

used to detect disseminated intravascular coagulation (DIC). Examination of blood smears, cytology smears, or bone marrow samples may reveal infectious organisms or neoplastic cells. Serology or polymerase chain reaction (PCR) testing is indicated to identify an infectious process. Flow cytometry is used to detect reticulated platelets, platelet surface-associated immunoglobulin (PSAIg), or activated platelets.⁷⁵ Reticulated platelets are immature platelets with increased amounts of RNA. Measurement of reticulated platelets may help differentiate thrombocytopenia due to shortened platelet survival from thrombocytopenia due to platelet production disorders. Reticulated platelets were increased in dogs and horses with destructive or consumptive thrombocytopenia.^{57,78} Identification of PSAIg may support a diagnosis of immune-mediated disease but these assays do not differentiate primary from secondary immune-mediated thrombocytopenia (IMT).^{31,78} Detection of activated platelets may identify animals that are in a prothrombotic state or in DIC.^{48,62}

Bone marrow examination may be useful if a platelet production problem is suspected. If thrombocytopenia is due to a production disorder, megakaryocytes should be decreased or totally absent. Neoplastic cells or etiologic agents may be identified. If thrombocytopenia is due to platelet destruction or consumption, megakaryocytes should be normal or increased in number. The value of bone marrow examination as an initial diagnostic assay in animals with thrombocytopenia is debatable. A recent study in thrombocytopenic dogs reported that bone marrow cytology provided useful diagnostic or prognostic information in a minority of cases, suggesting that bone marrow aspiration was most likely to be useful in animals with thrombocytopenia that was unresponsive to therapy or was accompanied by bicytopenia or pancytopenia.⁴⁶ Immunofluorescence assays may be used to detect anti-megakaryocyte antibodies on bone marrow cytology preparations; however, the sensitivity and specificity of these assays are low.⁶⁶

BASIC MECHANISMS FOR THROMBOCYTOPENIA

Thrombocytopenia occurs when platelets are removed from circulation faster than they are replaced from the bone marrow. This occurs because of decreased platelet survival or decreased platelet production. Basic mechanisms for decreased platelet survival include loss, distribution disorders, consumption with excessive thrombi formation, or destruction. Decreased platelet production results from impaired megakaryopoiesis or thrombopoiesis. Often multiple mechanisms are involved in the pathogenesis of thrombocytopenia in an animal.

Platelet Loss

Thrombocytopenia, usually mild to moderate in severity, may follow external hemorrhage. Platelet concen-

tration decreased by up to 50% in dogs with experimental acute, severe blood loss.⁶⁶ Thrombocytopenia due to blood loss is usually self-limiting and resolves when the hemorrhage resolves. When severe thrombocytopenia occurs in conjunction with hemorrhage, it is likely that the hemorrhage is due to thrombocytopenia and not that the thrombocytopenia is due to hemorrhage.

Dogs that have anticoagulant rodenticide toxicity frequently have thrombocytopenia and the thrombocytopenia may be severe.³⁹ Likely mechanisms for thrombocytopenia include loss associated with hemorrhage and platelet consumption during formation of hemostatic plugs at sites of hemorrhage.⁶⁶

Platelet Consumption

Accelerated platelet consumption occurs with disorders that cause widespread damage to endothelial cells or trigger massive activation of coagulation via other mechanisms. In people, well characterized platelet consumptive disorders include DIC and thrombotic microangiopathies such as thrombocytopenic thrombotic purpura (TTP) and hemolytic uremic syndrome (HUS).

Thrombotic Microangiopathies

Thrombotic microangiopathies are uncommon, life-threatening conditions characterized by disseminated thrombosis in the microvasculature, secondary ischemic organ damage, microangiopathic hemolytic anemia with schistocytes, and thrombocytopenia. Laboratory evidence of abnormalities in coagulation and fibrinolysis pathways is generally lacking.⁴² Thrombotic microangiopathies are associated with inherited or acquired disorders and the underlying pathogenesis is often heterogeneous.⁸⁰

Some people consider HUS and TTP to be variants of the same condition and differentiation of the two disorders is challenging.^{42,80} Based upon clinical presentation, HUS is associated with more severe renal insufficiency. The most common form of HUS occurs primarily in children and follows a period of infectious diarrhea, particularly involving enterohemorrhagic *Escherichia coli*. The atypical form of HUS in humans is often associated with inherited or acquired defects in complement regulation.^{26,49,80} Although rare, HUS syndromes have been described in dogs, cats, cattle, and horses.^{3,13,15,55}

When compared to HUS, the clinical presentation of TTP is associated with frequent neurologic dysfunction, less severe renal insufficiency, and evidence of microthrombi that are rich in platelets and VWF. Recent findings indicate that many people with TTP have inherited or acquired deficiency of ADAMTS13, a metalloprotease that cleaves VWF and prevents accumulation of the most thrombogenic, high relative molecular mass forms of VWF in the blood.^{49,80} TTP has not been well-documented in veterinary species; however, spontaneous TTP has been reported in pigs and there are animal models of experimentally induced TTP.^{43,59}

Disseminated Intravascular Coagulation

Unlike HUS and TTP, DIC is commonly diagnosed in veterinary species. Disseminated intravascular coagulation differs from HUS and TTP in that patients with DIC typically have laboratory evidence of abnormalities in coagulation and fibrinolytic pathways.⁴² DIC is never a primary condition; it occurs secondary to a wide variety of disorders that are characterized by excessive and unregulated coagulation. DIC varies from overt or uncompensated to non-overt or compensated. In non-overt DIC, excess thrombin is generated but is balanced by inhibitory pathways. In overt DIC, there is widespread microthrombi formation and bleeding may result from consumption of coagulation factors and platelets.^{37,67} Clinical signs vary from subclinical in non-overt DIC to signs of severe, life-threatening hemorrhage, shock or multiorgan failure in overt DIC. Clinical signs referable to the underlying condition are often present.⁶⁶ The consequences of hemorrhage are easily recognized; however, clinical signs and death are often attributable to organ dysfunction resulting from microthrombi formation and resulting tissue hypoxia.^{37,67}

The pathogenesis of DIC is further discussed in Chapter 88. Briefly, DIC is characterized by overwhelming activation of coagulation and subsequent disruption of the normal regulatory mechanisms. Widespread fibrin deposition results due to excessive thrombin generation, loss or inhibition of physiologic anticoagulants, impaired fibrinolysis, and release of proinflammatory cytokines.¹⁹ Triggers for thrombin generation generally include extensive vascular disruption or massive release of tissue factor from damaged tissue.⁶⁶ Snake venoms may directly activate coagulation factors. Specific disorders associated with DIC include infections (e.g. septicemia, endotoxemia), malignant neoplasia, severe tissue injury (e.g. trauma, surgery, burns), immune-mediated disorders (e.g. immune-mediated hemolytic anemia), conditions that cause blood stasis (e.g. shock, severe dehydration), heatstroke, systemic hypersensitivity reactions, pancreatitis, snakebites or hepatic failure.^{37,56,66}

Thrombocytopenia due to DIC varies from mild to severe. Unfortunately, no single test is diagnostic for DIC. Tests that detect markers of thrombin activation (e.g. prothrombin fragments 1+2, thrombin-antithrombin complexes) or fibrin generation (e.g. fibrinopeptide A) are sensitive; however, they are difficult to run and lack specificity.⁶⁷ The International Society on Thrombosis and Haemostasis proposed a diagnostic algorithm to diagnose overt DIC in people using platelet concentration, PT, fibrinogen concentration, and FDP or D-dimer. A more complex diagnostic algorithm adding evaluation of abnormal trends in coagulation screening data, AT activity and protein C activity has been proposed to detect non-overt DIC.⁷⁰

The diagnosis of DIC in veterinary species usually relies on identifying abnormalities involving multiple hemostatic pathways. Laboratory findings include evidence of thrombocytopenia, coagulation defects (pro-

longed APTT or PT, hypofibrinogenemia), enhanced fibrinolysis (increased FDPs or D-dimer), and loss of natural anticoagulants (e.g. decreased AT activity). Identification of schistocytes or keratocytes on a blood smear is also supportive.⁶⁶

Platelet Destruction

Immune-mediated Platelet Destruction

Immune mediated thrombocytopenia (IMT) is commonly diagnosed in dogs and sporadically recognized in other species. IMT occurs when antibodies bind to platelets and cause shortened platelet lifespan due to complement-mediated lysis or platelet removal by the mononuclear phagocytic system (MPS). Direct T cell mediated cytotoxicity as well as impaired megakaryopoiesis and thrombopoiesis are implicated in the pathogenesis of IMT.^{65,81}

With primary IMT, the immune response is directed against autoantigens and no underlying disease is identified. In people with primary IMT, the most common target antigens are located on integrin $\alpha_{IIb}\beta_3$.⁸¹ Target antigens are rarely identified in animals with primary IMT. Antibodies directed against $\alpha_{IIb}\beta_3$ and GPIb were detected in dogs with primary IMT.⁴⁰ With secondary IMT, the immune response is associated with an underlying disorder such as systemic immune-mediated diseases (e.g. immune-mediated hemolytic anemia), neoplasia (e.g. lymphoma, hemic neoplasia, various solid tumors), drug administration, and infections.^{40,66}

The diagnosis of IMT generally relies on exclusion of other causes of thrombocytopenia and response to immunosuppressive therapy. Detection of PSAIg is supportive but testing is not frequently performed in veterinary species.⁶⁶

The pathogenesis and diagnosis of IMT is covered in more depth in Chapter 78.

Non-immune-mediated Platelet Destruction

Non-immune platelet destruction occurs in disorders that cause platelet aggregation, phagocytosis, or lysis independent of antibody or complement. Non-immune platelet destruction is often a feature of thrombocytopenia due to infections, drugs, or neoplasia. These are disorders with complex mechanisms and are discussed separately below. Potential mechanisms for non-immune destruction include direct damage to platelets causing lysis or premature removal, release of substances that directly activate platelets, cytokine-mediated activation of the MPS leading to enhanced phagocytosis of platelets, hemophagocytic syndrome, or phagocytosis by neoplastic cells.^{1,66,76} Hemophagocytic syndrome is characterized by a non-neoplastic proliferation of activated macrophages and is associated with multiple cytopenias in the blood. Hemophagocytic syndrome occurs secondary to immune-mediated disorders, infections, and neoplasia. In some cases, an underlying condition cannot be identified.⁷⁶

Platelet Distribution Disorders

The total platelet mass in any animal consists of platelets freely circulating in the blood and platelets reversibly distributed in the vascular systems of tissues, particularly the spleen, liver and bone marrow. In some healthy animals, 30% or more of total platelet mass is stored in the spleen.⁸ Redistribution thrombocytopenia occurs when platelets become reversibly sequestered in tissues. Total platelet mass is not affected so increased thrombopoiesis is not expected. Disorders associated with increased platelet destruction are not considered redistribution disorders. Platelet pooling has been reported in the liver and spleen of dogs with hypothermia and in the lungs of dogs with endotoxemia.⁶⁶ Mild to moderate thrombocytopenia may result from sequestration in animals with any condition associated with splenomegaly or hepatomegaly and increased blood pooling.⁸

Clinical hemorrhage is not expected with platelet sequestration unless the underlying disorder is complicated by platelet dysfunction or a coagulation disorder. Clinical signs are generally limited to those associated with the underlying disorder. Diagnosis of redistribution thrombocytopenia relies on discovering the cause for organomegaly and ruling out other potential causes of thrombocytopenia.

Platelet Production Disorders

Bone marrow production disorders are covered in Chapters 16–19. Decreased platelet production may result from disorders that specifically target megakaryocytes or from diseases that have more generalized effects to impact production of multiple hematopoietic cell lines directly or by altering the microenvironment in the bone marrow. Bicytopenia or pancytopenia in the blood suggests generalized bone marrow disease.⁶⁶

Selective megakaryocytic hypoplasia is uncommon. Acquired immune-mediated amegakaryocytic thrombocytopenia has been reported in dogs and cats.^{35,83} Megakaryocytic hypoplasia can be associated with drugs, infections or neoplasia. Neoplasia may be metastatic to the bone marrow or hemic in origin, causing replacement of the normal hematopoietic cells. Other causes of hypoplasia include irradiation and myelonecrosis.⁶⁶ Hereditary causes of megakaryocytic hypoplasia are documented in people but have been rarely reported in veterinary species.^{5,32}

High quality bone marrow samples are required to adequately assess bone marrow production. Decreased megakaryocytes should be interpreted with caution in an aspirate, and core biopsy samples are recommended for definitive diagnosis of megakaryocytic hypoplasia.⁶³

Disorders with Complex Mechanisms

Thrombocytopenia has been frequently associated with infection, neoplasia and drug therapy in veterinary species.^{22,27,64} In many cases, the pathogenesis of the

thrombocytopenia is unclear and likely multifactorial in origin.

Infectious Thrombocytopenia

Thrombocytopenia frequently accompanies infections.^{22,25,27,64} Some of the organisms associated with thrombocytopenia are listed in Table 79.2. Many organisms impair platelet production in the bone marrow by direct infection of megakaryocytes, stimulation of an immune response targeting megakaryocytes, or stimulation of an inflammatory response in the bone marrow and production of myelosuppressive cytokines. Platelet survival is often decreased. Proposed pathophysiologic mechanisms include increased platelet consumption due to platelet activation and associated DIC, direct infection of platelets causing destruction or removal by the MPS, secondary IMT, or platelet sequestration in the spleen or other tissues.^{56,66}

With endotoxemia or some bacterial infections, platelets are activated due to a direct effect of endotoxins or inflammatory cytokines.⁶¹ Inflammation leads to production of platelet stimuli (e.g. thrombin, platelet activating factor) by activated endothelial cells, neutrophils, monocytes and platelets.^{61,72} Activated platelets enhance tissue factor expression by monocytes, leading to activation of the coagulation cascade.^{54,61} Endothelial cells become injured and consequently decrease production of inhibitory substances such as prostaglandin I₂. Activated platelets express P selectin which plays a role in platelet adhesion to neutrophils and monocytes.⁷⁷ Inflammatory cytokines mediate activation of the MPS which may further enhance phagocytosis of platelets.¹

Rickettsial bacteria are commonly associated with thrombocytopenia. Acute infection with *Ehrlichia canis* is associated with immune-mediated and non-immune platelet destruction in dogs.⁷⁴ Chronic infection is associated with bone marrow hypoplasia.⁶⁶ Other rickettsial bacteria associated with thrombocytopenia include *Anaplasma platys* in dogs, *Rickettsia rickettsii* in dogs, *Neorickettsia risticii* in horses, and *Anaplasma phagocytophilum* in several species.^{18,41} *A. phagocytophilum*, the cause of granulocytic anaplasmosis (granulocytic ehrlichiosis), was reported to directly infect megakaryocytes but to not alter platelet production. Immune responses were implicated in the pathogenesis of the thrombocytopenia.²¹ The pathogenesis of thrombocytopenia in *A. platys* infection is unknown but may involve sequestration or removal of infected platelets by macrophages. In experimentally infected dogs, PCR testing was positive for the organism in the spleen and bone marrow.¹⁶ Vasculitis and platelet consumption occur in *R. rickettsii* infection and may be the primary causes for thrombocytopenia. The cause of thrombocytopenia in *N. risticii* infection is unclear but likely involves consumption due to DIC.⁵⁶ Other bacterial infections (e.g. leptospirosis, salmonellosis, borreliosis, bartonellosis) are sporadically associated with thrombocytopenia.^{6,18,56,66}

Viral infections are well-recognized causes of thrombocytopenia in animals. In dogs, thrombocytopenia

TABLE 79.2 Reported Causes of Thrombocytopenia in Dogs, Cats, Horses and Cattle

Species	General Category	Specific examples ^a
Dog	Infectious	Viruses: distemper, herpes virus, infectious hepatitis, parvovirus Bacteria: septicemia, endotoxemia, <i>Anaplasma phagocytophilum</i> , <i>A. platys</i> , <i>Ehrlichia canis</i> , <i>Rickettsia rickettsii</i> , leptospirosis, salmonellosis, bartonellosis, borreliosis Fungi: histoplasmosis, candidiasis Protozoa: babesiosis, leishmaniasis Parasites: heartworm disease
	Neoplasia	Hemangiosarcoma, lymphoproliferative and myeloproliferative neoplasia, various solid tissue neoplasms
	Drugs/toxins	Antibiotics (e.g. sulfonamides, cephalosporin); chemotherapeutic drugs (e.g. cyclophosphamide, doxorubicin), anti-inflammatory drugs (e.g. phenylbutazone, carprofen), phenobarbital, thiacetarsamide, estrogen, snakebites
	Miscellaneous	IMT, DIC, distribution disorders
Cat	Infectious	Viruses: FeLV, FIV, FIP, parvovirus Bacteria: septicemia, endotoxemia, <i>A. phagocytophilum</i> , <i>Mycoplasma</i> sp., salmonellosis, bartonellosis Fungi: histoplasmosis Protozoa: cytauxzoonosis, toxoplasmosis Parasites: heartworm disease
	Neoplasia	Lymphoproliferative and myeloproliferative neoplasia, various solid tissue neoplasms
	Drugs/toxins	Chemotherapeutic drugs, griseofulvin, methimazole, propylthiouracil
	Miscellaneous	IMT, DIC, distribution disorders
Horse	Infectious	Viruses: EIA, EVA, VEE Bacteria: septicemia, endotoxemia, <i>A. phagocytophilum</i> , <i>Neorickettsia risticii</i> , salmonellosis
	Neoplasia	Lymphoproliferative and myeloproliferative neoplasia, various solid tissue neoplasms
	Drugs/toxins	Phenylbutazone, trimethoprim, penicillin, trichothecene mycotoxin, snakebites
	Miscellaneous	IMT, neonatal alloimmune thrombocytopenia, DIC, gastrointestinal disorders, distribution disorders
Cattle	Infectious	Viruses: BVD Bacteria: septic metritis/mastitis, septicemia, endotoxemia, salmonellosis Protozoa: babesiosis, theileriosis
	Drugs/toxins	Bracken fern toxicity, trichothecene mycotoxin
	Miscellaneous	IMT, DIC, distribution disorders, displaced abomasums

^aBVD, bovine viral diarrhea; DIC, disseminated intravascular coagulation; EIA, equine infectious anemia; EVA, equine viral arteritis; FeLV, feline leukemia virus; FIV, feline immunodeficiency virus; FIP, feline infectious peritonitis; IMT, immune-mediated thrombocytopenia; VEE, Venezuelan equine encephalitis.

occurs with canine distemper virus (CDV), herpes virus, infectious hepatitis, or parvovirus infection. The thrombocytopenia in CDV infection results from viral damage to platelets, secondary IMT, and decreased production due to direct infection of megakaryocytes.⁴ Thrombocytopenia associated with herpes virus or infectious hepatitis virus infection results from endothelial damage and DIC.⁶ In cats, thrombocytopenia is associated with feline infectious peritonitis (FIP), feline leukemia virus (FeLV), feline immunodeficiency virus (FIV), and parvovirus infection. Thrombocytopenia in FIP is multifactorial and results from vasculitis, secondary IMT, direct viral damage to platelets, and DIC.⁷ In horses, thrombocytopenia occurs with equine infectious anemia (EIA), equine viral arteritis, and Venezuelan encephalitis virus infection. Thrombocytopenia in EIA results from cytokine mediated impairment of platelet production, secondary IMT, and non-immune platelet destruction.^{10,58} In cattle, thrombocytopenia is associated with bovine viral diarrhea virus and primarily results from megakaryo-

cyte damage and subsequent decreased platelet production.⁷³ In pigs, thrombocytopenia follows infection with African swine fever or classical swine fever (hog cholera). In classical swine fever, thrombocytopenia results from direct damage to platelets, platelet activation and removal by the MPS, DIC, and impaired megakaryopoiesis.^{20,52}

Other agents associated with thrombocytopenia include protozoa (e.g. babesiosis, cytauxzoonosis, leishmaniasis, theileriosis, and toxoplasmosis), fungi (e.g. disseminated candidiasis, histoplasmosis), and metazoan parasites (e.g. heartworm infection in dogs and cats).^{6,22,27,56,66}

Infection-induced thrombocytopenia is often suspected based upon history and clinical findings. Thrombocytopenia varies from mild to severe. Microscopic examination of blood, bone marrow or cytology smears may identify infectious agents. If organisms are not visible microscopically, serology, culture, or molecular (e.g. PCR) techniques may be diagnostic.

Drug-induced Thrombocytopenia

Thrombocytopenia is often suspected following exposure to drugs or toxins. Some drugs associated with thrombocytopenia in veterinary species are included in Table 79.2. Drug-induced blood cell disorders are covered in Chapter 16 and only a brief summary is provided here. With some drugs (e.g. chemotherapeutic drugs), thrombocytopenia is dose-dependent and predictable.^{23,66} Chemotherapeutic-induced thrombocytopenia is usually transient and resolves when the drug is withdrawn or dosage is altered. With other drugs (e.g. potentiated sulfas), thrombocytopenia is an idiosyncratic and unpredictable result.⁷¹

Many drugs are known to have myelosuppressive effects. Secondary IMT, targeting either platelets or megakaryocytes, is frequently implicated as a mechanism for drug-induced thrombocytopenia. In most cases, the immune response occurs only when the drug or drug metabolite is present.⁸² Antibodies may target the drug or metabolite adsorbed to the platelet membrane, a complex formed between the drug and a platelet antigen, or a hidden antigen exposed on the platelet membrane.^{66,82} Subsequent binding of antibody targets the platelet for removal by the MPS. Rarely, drugs cause IMT that is drug-independent and persists after the drug is removed. These types of drugs appear to stimulate an immune response that targets unaltered platelet antigens.^{66,82} Drug-induced IMT is often difficult to prove in these situations. PSAIg has been detected in dogs following administration of sulfonamides and in horses following administration of trimethoprim and penicillin.^{36,45}

Some drugs directly activate platelets causing increased consumption, or injure platelets causing removal by the MPS. Snake venoms may either directly activate platelets or may cause the production of substances such as thrombin that cause platelets to aggregate.²⁴

Diagnosis of drug associated thrombocytopenia generally relies on a history of thrombocytopenia occurring following drug administration and normalization of platelet concentration following cessation of drug therapy.

Neoplasia Associated Thrombocytopenia

Thrombocytopenia is commonly attributed to neoplasia.^{22,23,27,63} In dogs with neoplasia, thrombocytopenia was detected in 10–36% of affected animals.⁸ Lymphoma, carcinoma, hemangiosarcoma, and hematopoietic neoplasia comprised the majority of cases. Thrombocytopenia was frequently attributed to chemotherapy, decreased platelet production, or DIC.²³

Decreased platelet production may result from myelophthisis, secretion of estrogen by neoplastic cells or myelosuppressive chemotherapeutic drugs.⁸ Disseminated intravascular coagulation is a frequent complication of neoplasia. In one study, over 10% of dogs with malignant neoplasia had DIC. Hemangiosarcoma, mammary carcinoma and adenocarcinoma of

the lung were at greatest risk.⁴⁴ Neoplasia is the most commonly recognized cause of DIC in cats.¹⁷

Secondary IMT has been implicated as a mechanism for thrombocytopenia in a variety of hemic and non-hemic neoplasias.^{8,34,63} Other proposed mechanisms for neoplasia-induced thrombocytopenia include distribution disorders with platelet pooling (particularly with large vascular tumors), platelet loss with tumor associated hemorrhage, or platelet destruction by neoplastic cells.^{8,66} Thrombocytopenia was identified in 88% of dogs with hemophagocytic histiocytic sarcoma and phagocytosis of platelets by neoplastic cells was believed to play a role.⁴⁷

Clinical signs are often related to the underlying neoplasm; thrombocytopenia may or may not be severe enough to cause a defect in primary hemostasis. Diagnosis is based upon cytologic or histopathologic identification of neoplastic cells. If the neoplasia is hemic in origin, then abnormal cells may be present in the blood.

Miscellaneous Causes of Thrombocytopenia

Breed Associated Thrombocytopenia

Thrombocytopenia is an incidental finding in certain dog breeds. Affected dogs are healthy and do not have evidence of a bleeding disorder. Greyhounds often have thrombocytopenia when compared to non-breed specific reference intervals. Platelet concentrations are typically greater than 100,000/ μ L. In one study, none of the samples tested had detectable PSAIg, suggesting that the thrombocytopenia is not immune-mediated in origin.⁶⁰ An inherited thrombocytopenia occurs in approximately 50% of Cavalier King Charles Spaniels (see Chapter 82). Macrothrombocytes are present in many dogs, making automated platelet counts unreliable.⁹ Recent studies suggest that there is a mutation in the gene encoding beta1-tubulin, leading to unstable microtubule formation in megakaryocytes and altered proplatelet formation.¹²

Pseudothrombocytopenia

Pseudothrombocytopenia occurs when platelets in a sample are excluded from counting for any reason. Decreased automated platelet concentration should always be confirmed by microscopic examination of a blood smear. Small clumps may not be detected by automated analyzers and still falsely lower measured platelet concentrations.⁶⁸ A recent study in cats reported that platelet concentrations determined with an impedance hematology analyzer were decreased in a majority of animals tested; however, examination of a blood smear confirmed thrombocytopenia in only 3.1% of cats. The disparity was attributed to the presence of platelet clumps and inability to separate platelets and erythrocytes based upon size.⁵¹ When clumps are detected in a sample, the measured platelet concentration is of minimal value and collection of a new blood sample without clumping is required to obtain an accu-

rate platelet concentration. Platelet concentration determined by analyzers that use a buffy coat technique appear to be less affected by platelet clumping than impedance hematology analyzers.³³

Proper blood collection and handling are critical to minimize the clumping that occurs when platelets become activated and form aggregates. Clumping can be minimized by atraumatic venipuncture or addition of platelet inhibitors to the sample.⁵⁰ Platelet clumps may form in vitro in an EDTA-dependent manner. In people, EDTA causes a conformational change in $\alpha_{IIb}\beta_3$ that exposes normally hidden epitopes that bind to circulating antibodies. EDTA-dependent pseudothrombocytopenia has rarely been reported in dogs, horses and pigs; however, the underlying mechanism has not been determined.⁷⁹

Samples with increased megaplatelets may have falsely low platelet concentrations because large platelets are excluded by some automated hematology analyzers. In these animals, manual platelet counts using a hemocytometer and microscope may provide a more accurate platelet concentration.⁵³

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Essential Thrombocythemia and Reactive Thrombocytosis

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Thrombopoiesis: A Brief Review
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Acronyms and Abbreviations

CIMF, Chronic idiopathic myelofibrosis; CML, chronic myeloid leukemia; CMPD, chronic myeloproliferative disease; ECMP, European clinical, molecular and pathological criteria; ET, essential thrombocythemia; GM-CSF, Granulocyte monocyte colony stimulating factor; IL, interleukin; JAK2, Janus kinase 2; MCV, mean cell volume; PV, Polycythemia vera; PVSG, Polycythemia Vera Study Group; SD, standard deviation; SDF-1, stromal cell-derived factor-1; STAT, signal transducer and activator of transcription; TPO, thrombopoietin; WHO, World Health Organization.

Thrombocytosis, or thrombocythemia, is defined as a high platelet count or, more specifically, a count that is above a reference interval established for the species. Various methods are available for measuring platelet counts, including estimates from stained peripheral blood smears, manual hemocytometer counts, quantitative buffy coat analysis, and automated methods based on impedance or laser-induced light scatter. These vary in precision and accuracy and results from the same animal may differ between methods.^{31,59} Therefore, most veterinary laboratories establish reference intervals specific for their own equipment for routinely analyzed species. For those animals in which laboratory-specific intervals are unavailable, interpretation of the platelet count is more difficult. Knowledge of the normal physiology of the animal, combined with published reference intervals, can be used as a guide to data interpretation. Platelet counts in healthy animals vary markedly between species. For instance, small rodents normally have very high counts, whereas horses have relatively low counts (Table 80.1). Thus, a normal platelet count in a mouse would be regarded as an extreme thrombocytosis in a dog or horse.

A potentially misleading consequence of thrombocytosis is a pseudohyperkalemia that occurs in serum samples. This is due to release of intracellular potassium during clotting and has been most thoroughly evaluated in the dog.^{13,49} Potassium concentrations can be 0.63 ± 0.17 mEq/L (mean \pm SD) higher in serum than plasma in dogs with platelet counts between 150,000–600,000/ μ L.⁴⁹ This difference increases in dogs with higher counts (1.55 ± 0.73 mEq/L) and can be quite marked (up to 5 mEq/L) in dogs with extreme thrombocytosis ($>1,000,000$ platelets/ μ L).^{3,13,25,39,49} To avoid this artifact, heparinized plasma is the preferred sample for potassium measurement.

Thrombocytosis can be due to an artifact (pseudothrombocytosis), altered trafficking of platelets (i.e. release from storage pools; also called physiologic) and increased platelet production (enhanced thrombopoiesis). Enhanced thrombopoiesis can be secondary to drugs, cytokines (also called reactive), clonal hematopoietic disorders, or familial/inherited megakaryocyte disorders (Table 80.2).

To understand the pathophysiology of thrombocytosis, the factors governing thrombopoiesis are briefly reviewed below (see Chapter 9 for more details).

THROMBOPOIESIS: A BRIEF REVIEW

Platelets are produced from megakaryocytes in the bone marrow, by a unique process that involves fragmentation of long cytoplasmic extensions (proplatelets) that the megakaryocyte inserts between bone marrow sinusoidal endothelial cells. The precise mechanisms controlling platelet release from proplatelets are largely unknown, however this process does require adhesion of megakaryocytes to subendothelial matrix compo-

TABLE 80.1 Reference Intervals for Platelet Counts from Various Animal Species

Species	Reference Interval (range or mean \pm SD) ($\times 10^3/\mu\text{L}$)
Canine	186–545 ^a
Feline	195–624 ^a
Equine	94–246 ^a
Bovine	252–724 ^a
Alpaca	220–947 ^a
Caprine	245–975 ^b
Ovine	250–750 ^c
Porcine	520 \pm 195 ^c
Mice	1163 \pm 382 ^{c,d}
Long Evans Rats (13–15 months)	993 \pm 183 ^{c,d}

^aIntervals established for the 2120 ADVIA hematology analyzer at Cornell University (2008).

^bIntervals established for the 120 ADVIA hematology analyzer at Cornell University (2000).

^cRef. 28.

^dSex- and strain-dependent differences apply.

nents (particularly fibronectin and thrombospondin), cytoskeletal elements (actin, microtubules), pro-apoptotic enzymes,¹² matrix metalloproteinases³³ and shear forces (which facilitate proplatelet fragmentation). To date, the only known soluble mediator of platelet release is the chemokine, stromal cell-derived factor-1 (SDF-1);³³ none of the known thrombopoietic cytokines, including thrombopoietin (TPO), appear to stimulate this process.³⁵ It has been estimated that approximately 2,000–3,000 platelets are produced from each megakaryocyte, with the residual megakaryocyte nucleus being degraded by phagocytes within the bone marrow.³⁵

Thrombopoietin is the main thrombopoietic cytokine and is produced primarily in the liver and kidney. TPO binds to its receptor, MPL (a product of the proto-oncogene, *c-mpl*) to mediate downstream responses through the non-receptor tyrosine kinase, Janus kinase 2 (JAK2), which in turn activates the transcription factors signal transducer and activator of transcription (STAT 3 and 5).^{29,35} MPL is highly expressed on megakaryocytes and platelets, but is also expressed on other cell types, including hematopoietic stem cells. TPO levels in serum are thought to be regulated by binding to MPL, with subsequent internalization and degradation. TPO mainly acts as a differentiation factor, stimulating stem cells to differentiate along the megakaryocytic lineage and preventing apoptosis through the protein Bcl-XL.³² It is also required for complete megakaryocyte maturation. TPO acts in concert (additively or synergistically) with other hematopoietic cytokines, including stem cell factor, interleukin-3 (IL-3) and erythropoietin, to promote megakaryocyte proliferation and polyploidy.

TABLE 80.2 Causes and Associated Mechanisms of Thrombocytosis^{23,51,54,56}

Cause	Mechanism
Pseudothrombocytosis	
Red cell ghosts or fragments, fragile leukocytes	Non-platelet cell fragments are counted as platelets
Physiologic	
Epinephrine (trauma, exercise, excitement)	Splenic contraction
Post-splenectomy	Lack of sequestration
Drug-induced	
Epinephrine	Splenic contraction
Vincristine	Enhanced megakaryopoiesis
Reactive	
Inflammation, infection, neoplasia (hematopoietic and non-hematopoietic), trauma, rebound from thrombocytopenia	Cytokine-mediated (directly or indirectly through TPO production) enhanced megakaryopoiesis
Iron deficiency-related	Unknown
Neoplasia involving megakaryocytes	
Chronic myeloproliferative disease: essential thrombocythemia, chronic myeloid leukemia, chronic basophilic leukemia, polycythemia vera, chronic idiopathic myelofibrosis	Acquired or inherited genetic mutations causing TPO-independent proliferation, e.g. JAK2 signaling mutations
Acute megakaryocytic leukemia	Acquired or inherited genetic mutations
Hereditary/familial ^a	Inherited genetic mutations

^aNot reported in animals.

Other thrombopoietic cytokines are SDF-1,³⁸ IL-6 superfamily members (IL-6, IL-11, leukemia inhibitory factor), GM-CSF, IL-1 α and IL-9.^{29,35}

Thus, potential mechanisms that would result in thrombocytosis due to megakaryopoiesis are increased concentrations of thrombopoietic cytokines, ligand-independent MPL activation, and abnormal activation of the JAK2/STAT signaling pathway.

CAUSES OF THROMBOCYTOSIS

Pseudothrombocytosis

Pseudothrombocytosis can occur when small, fragmented or hemolyzed erythrocytes, leukocyte fragments (from normal or leukemic cells), or particulate cellular debris are erroneously counted as platelets (Fig. 80.1). Although this artifact can be analyzer-dependent,

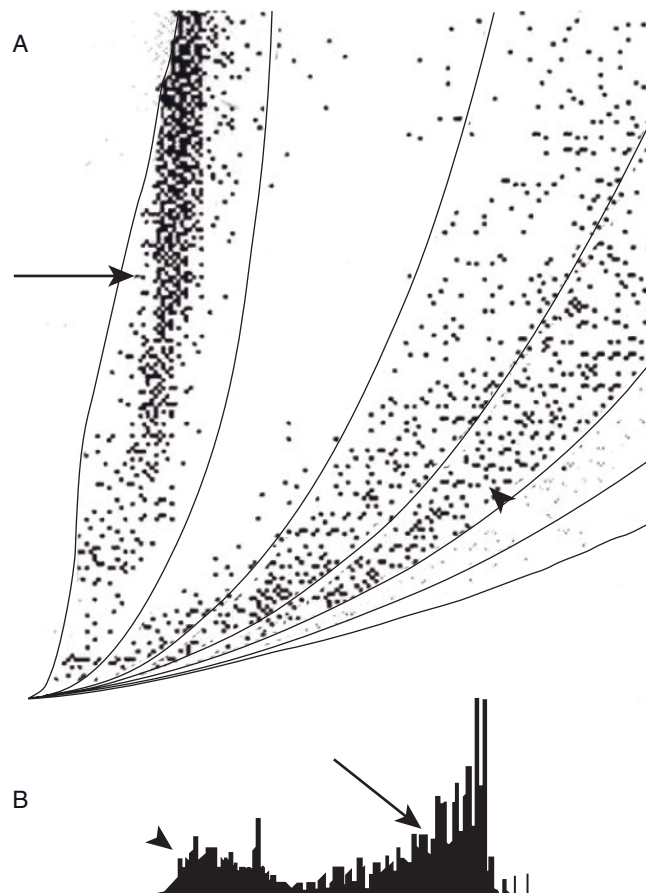


FIGURE 80.1 Pseudothrombocytosis in a hemolyzed sample from a dog with immune-mediated hemolytic anemia. (A) Hemolyzed red cells (arrow) can be seen entering the upper left portion of the platelet cytogram (forward scatter versus side scatter dotplot) and are counted as platelets on the ADVIA 120 hematology analyzer (laser light scatter-based system). (B) The hemolyzed erythrocytes can also be visualized as a second population of macrocytic cells (arrow) in the platelet histogram (cell number versus cell volume) and will artificially increase the mean platelet volume and platelet distribution width. The “true” platelet clusters are identified by the arrowhead in both cytograms.

it can potentially occur with any counting method. Thus, it is important to always verify platelet counts by estimating numbers from stained blood smears.^{21,58} Although estimates are of questionable accuracy, smear examination can quickly confirm an elevated platelet count, identify morphologic defects in platelets and potentially reveal abnormalities that may cause a pseudothrombocytosis.

Physiologic Thrombocytosis

Up to one-third of the platelet mass is normally sequestered in the splenic red pulp. These stores can be released upon splenic contraction, which is mediated by epinephrine.^{34,65} A transient or persistent thrombocytosis may occur post-splenectomy in humans,⁵³ however this is not a consistent finding in dogs.⁵⁰ Although some authors have suggested the presence of an adrenaline-responsive, platelet pool in the lungs,²⁰ there is no definitive evidence for this.³⁴

Drug-induced Thrombocytosis

Two retrospective studies have made associations between various drugs and thrombocytosis in dogs and cats, however a direct cause and effect has not been established.^{23,56} It is possible that the thrombocytosis documented in animals on drug therapy is secondary to their underlying disease or a rebound from thrombocytopenia, rather than a drug response per se. Drugs documented to cause a thrombocytosis in animals are vincristine and epinephrine.^{34,37} Vincristine likely functions through enhanced megakaryopoiesis, whereas epinephrine induces a rapid (within a few minutes), transient (less than 60 minutes) thrombocytosis in dogs through splenic contraction, although there is large inter-animal variability in the response.^{20,34} Data for glucocorticoids are conflicting. Although hyperadrenocorticism is associated with thrombocytosis in dogs,¹⁹ two studies have not shown a consistent increase in platelet counts in healthy dogs given daily doses of up to 2 mg/kg prednisolone.^{37,46}

Reactive Thrombocytosis

Reactive thrombocytosis (or secondary thrombocytosis) is due to cytokine stimulation of thrombopoiesis. It is the most common cause of thrombocytosis in humans and animals and occurs secondary to various disorders (Table 80.2).^{6,22,23,51,54,56} Several retrospective studies in animals have shown that inflammation and neoplasia are the most common diseases associated with a thrombocytosis, although different platelet counts were used to define a thrombocytosis in these studies.^{23,51,54,56} A recent unpublished study at Cornell University further showed that 61% of 42 cats with a thrombocytosis (>750,000 platelets/ μ L) had a primary inflammatory or infectious disorder. Reactive thrombocytosis can be transient or persistent, depending on the underlying cause, so documenting a thrombocytosis more than

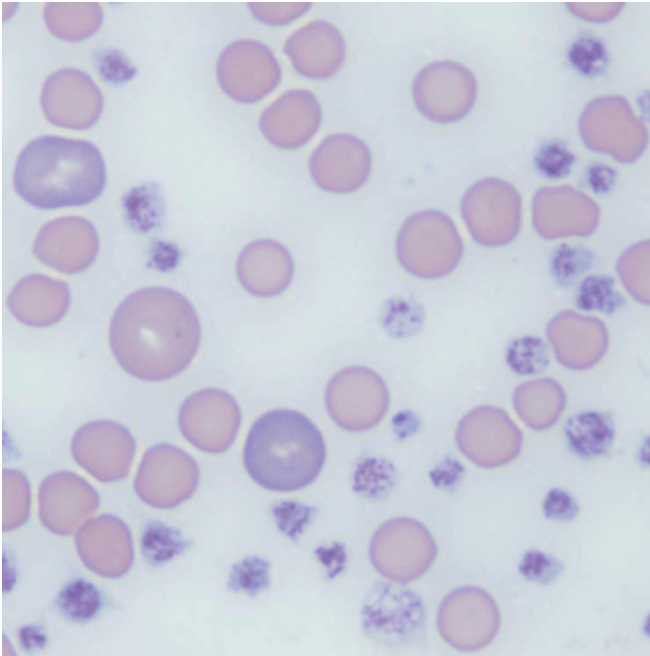


FIGURE 80.2 Peripheral blood smear from a 12 week old kitten with iron deficiency anemia secondary to flea infestation. Many platelets, which vary markedly in size, are seen in the smear. The kitten had an extreme thrombocytosis (platelet count = $1.9 \times 10^6/\mu\text{L}$), which was presumably reactive. Wright's stain; 1000 \times magnification.

once (particularly if done less than 1–2 months apart) does not exclude a reactive condition.⁵² Also, the increase in platelet count can be quite marked in reactive conditions ($>1,000,000/\mu\text{L}$) (Fig. 80.2).

There are a plethora of cytokines potentially responsible for a reactive thrombocytosis, including TPO, IL-6, GM-CSF, IL-3, IL-11 and erythropoietin. These can be constitutively secreted by tumors^{26,43} or secretion can be induced from host-derived cells as a result of inflammation, infection or neoplasia.⁵ The inflammatory cytokine IL-6 is considered one of the main mediators of a reactive thrombocytosis. IL-6 levels are increased in patients with thrombocytosis due to inflammation and neoplasia^{5,8,61} and persistently high levels are supportive of a reactive thrombocytosis.^{1,61} Rather than acting directly on megakaryocytes, IL-6 is thought to stimulate platelet production indirectly through induction of TPO production from hepatocytes.³⁰ Unfortunately, in some disorders typically associated with thrombocytosis (e.g. iron deficiency), the precise mechanism for the thrombocytosis is unknown.¹¹

The main differential diagnosis for reactive thrombocytosis in animals is essential thrombocythemia (ET). However, there are only a handful of reported cases of ET in dogs and cats, indicating that thrombocytosis in most animals (like humans) is likely reactive.

Essential Thrombocythemia in Humans

Essential thrombocythemia (also known as primary thrombophilia, hemorrhagic thrombocythemia/throm-

bocytosis, primary thrombocythemia and idiopathic thrombocythemia), is a clonal disorder of hematopoiesis, that primarily affects megakaryocytes. It is classified as a chronic myeloproliferative disease (CMPD), along with chronic myeloid leukemia (CML), polycythemia vera (PV), and chronic idiopathic myelofibrosis (CIMF), by the World Health Organization (WHO).²⁷ ET is the most frequently diagnosed CMPD^{22,27,52} and usually affects the elderly (>50 years), with no sex predilection. There is a second peak of incidence in females around 30 years old.^{27,52} Most patients (up to two-thirds) are asymptomatic at diagnosis, but affected individuals can paradoxically suffer from microvascular thrombosis (which is more common) or hemorrhage. Thrombi predominantly occur in arteries supplying the brain, heart, and extremities.^{27,52} The duration, but not degree, of thrombocytosis has been linked to a higher risk of thrombosis. The mechanism for thrombosis is largely unknown, but may involve interactions between activated platelets and leukocytes.² In contrast to thrombosis, hemorrhage is associated with an extreme thrombocytosis ($>1,000,000$ platelets/ μL), although hemorrhage can occur in patients with lower counts.^{52,63} Hemorrhage is typically from the skin or mucosa and has been attributed to acquired type II von Willebrand disease (VWD), with a selective loss of the high molecular weight multimers of von Willebrand factor (due to their preferential adsorption to platelets or enhanced proteolysis).^{41,52}

Essential thrombocythemia is characterized by a marked thrombocytosis in peripheral blood and a megakaryocytic hyperplasia in the bone marrow.²⁷ Leukocyte and erythrocyte counts are generally within normal limits. Platelets can vary markedly in size, but are usually not dysplastic. A histologic hallmark of ET is the presence of giant hyperlobulated megakaryocytes, found diffusely or in small loose clusters, within a normo- to slightly hypercellular marrow. Other evidence of dysplasia is lacking and there is no to minimal reticulin fibrosis.^{27,63} The main differential diagnoses for ET are reactive thrombocytosis (which is far more common) and other CMPDs (see below).

The discovery of a mutation in the JAK2 gene has advanced our understanding of the pathogenesis of ET and related CMPDs (PV and CIMF). JAK2 is a non-receptor tyrosine kinase that has a critical role in megakaryocyte and other hematopoietic cell signaling. JAK2 upregulates anti-apoptotic proteins, is required for efficient trafficking of MPL, and induces hematopoietic cytokine (e.g. IL-3, TPO) production.^{15,52} The mutation causes a phenylalanine for valine substitution at residue 617 (V617F) of the autoinhibitory domain and results in constitutive activation of JAK2.^{52,63,64} The V617F mutation is not specific for ET and also occurs in PV (up to 90% incidence) and CIMF, perhaps explaining the phenotypic and biologic similarities between these CMPDs.^{42,63,64} Constitutive JAK2 activation may explain the demonstrated hypersensitivity of megakaryocyte colonies derived from ET patients to thrombopoietic cytokines and to their autonomous growth in vitro.^{15,63,64} Testing for the JAK2 V617F mutation has

TABLE 80.3 Diagnostic Criteria for Essential Thrombocythemia in Humans

	PVSG ^a	WHO ^b	Revised WHO ^c
Positive criteria	Platelet count $\geq 600,000/\mu\text{L}$	Sustained platelet count $\geq 600,000/\mu\text{L}$ ^d Megakaryocytic hyperplasia (enlarged, mature)	Sustained platelet count $\geq 450,000/\mu\text{L}$, ^e large or giant platelets Megakaryocytic hyperplasia (enlarged, mature), no significant increase or left shifted neutrophil granulopoiesis or erythropoiesis, JAK2 mutations or other clonal markers
Exclusions			
Reactive thrombocytosis	No underlying disease	No underlying disease or prior splenectomy	No underlying disease
Iron deficiency	Stainable iron in marrow, failure of 1 month iron trial (to raise red cell mass)	Stainable marrow iron, normal ferritin, normal MCV (if these criteria not met, failure of iron trial)	Failure of iron trial in presence of decreased ferritin
Polycythemia vera	Normal red cell mass or hemoglobin	Normal red cell mass or hemoglobin	Normal hematocrit or hemoglobin
Chronic myeloid leukemia	No Philadelphia chromosome	No Philadelphia chromosome or BCR/ABL fusion	No Philadelphia chromosome or BCR/ABL fusion
Chronic idiopathic myelofibrosis	Collagen fibrosis absent or $< 1/3$ biopsy No splenomegaly No leukoerythroblastic reaction	Collagen fibrosis absent, reticulin fibrosis minimal or absent, no dysplasia	No leukoerythroblastic reaction, absence of relevant reticulin or collagen fibrosis, no abnormal megakaryocyte morphology or clusters
Myelodysplastic syndrome		No dysplasia No MDS-related gene defects	No dysplasia

^aRef. 47.^bRef. 27.^cRef. 62.^dSustained not defined. One recommendation is more than two occasions at least 2 months apart.⁵²^eSustained defined as during the work-up period.

been added to a proposed diagnostic scheme for ET⁴² (Table 80.3). However, since the mutation is only identified in up to 57% of patients with ET, non-JAK2 mutations are likely also involved in ET pathogenesis.^{15,48}

Essential thrombocythemia is considered an indolent disease, with affected patients having normal to slightly reduced lifespan. Unlike CML, ET rarely evolves into acute leukemia, but can transform into CIMF.^{42,52,63,64} Thrombohemorrhagic symptoms contribute to morbidity and mortality in ET patients and, consequently, cytoreductive therapy is recommended for patients likely to develop these complications. Such therapy includes platelet apheresis, hydroxyurea, anagrelide (a quinazoline derivative that inhibits megakaryocyte proliferation and differentiation) and interferon- α . Aspirin is frequently concurrently administered to patients at risk for thrombosis.^{40,60} Newer drugs targeting generic or JAK2 kinases may have promise in treating ET.⁴⁰

Differentiating Essential Thrombocythemia from Reactive Thrombocytosis

In humans, various laboratory and clinical criteria have been developed to try and distinguish reactive throm-

bocytosis from ET (Table 80.4). Although platelet counts trend higher in ET, there is substantial overlap between these two conditions and the degree of increase in the platelet count cannot be relied upon to distinguish between them.^{22,53} A diagnosis of ET over reactive thrombocytosis is favored by a history of thrombohemorrhagic events, mild to moderate non-progressive splenomegaly (due to extramedullary hematopoiesis), no evidence of inflammation, abnormally large megakaryocytes in the bone marrow, and identification of JAK2 or MPL mutations.^{42,52}

Differentiating Essential Thrombocythemia from other Chronic Myeloproliferative Disorders

Thrombocytosis is a common feature of all CMPDs, although the incidence and degree of increase differs between them.⁶³ Furthermore, there is a substantial overlap in clinical presentation, biology, and laboratory features between these diseases. In 1986, the Polycythemia Vera Study Group (PVSG) developed diagnostic criteria for ET. The diagnosis was one of exclusion; i.e. ruling out reactive thrombocytosis (particularly due to iron deficiency), PV and CML

TABLE 80.4 Criteria for Differentiating Between Reactive Thrombocytosis and Essential Thrombocythemia in Humans^{52,63}

Criteria	Reactive Thrombocytosis	Essential Thrombocythemia
Clinical		
Underlying disease	Yes	No
Thrombosis or hemorrhage	No ^a	Yes
Splenomegaly	No	Mild to moderate (due to extramedullary hematopoiesis)
Laboratory		
Platelet morphology	Normal	Normal, may see hypogranular/giant platelets
Bone marrow ^b	Normo- to hypercellular (concurrent granulocytic hyperplasia) Megakaryocytic hyperplasia (normal size, morphology)	Normocellular megakaryocytic hyperplasia (large cells, hyperlobulated or "staghorn" nuclei seen in loose clusters) ^b
Inflammatory markers (IL-6, fibrinogen, C-reactive protein)	High (may be normal)	Normal (may be high)
TPO levels	High or normal	High or normal ^c
Platelet aggregation	Normal	Abnormal (particularly to epinephrine) in 63–100%

^aMay occur if initiated by underlying disease.

^bAlthough these bone marrow changes are emphasized in the WHO classification scheme for ET, they may be quite subtle and are not present in all patients.

^cHigh TPO levels in ET have been attributed to down-regulation of MPL on platelets and megakaryocytes.

(Table 80.3).⁴⁷ In 2001, the WHO established a diagnostic scheme for ET. Like the PVSG criteria, this emphasized ET as a diagnosis of exclusion, but added bone marrow histopathology to exclude early or prefibrotic CIMF.^{27,63} However, it is now recognized that the WHO scheme excludes approximately 30% of ET patients with platelet counts below the designated cut-off (600,000/ μ L).^{42,63} For this reason, a new revised WHO has been proposed (Table 80.3). This uses a lower platelet cut-off and incorporates testing for JAK2 and MPL mutations.⁶² It remains to be seen whether this new scheme has any advantages over currently used criteria. Indeed, questions have been raised about the validity of the revised scheme, emphasizing the difficulty in differentiating the various CMPDs.⁵⁷

Essential Thrombocythemia in Animals

There are only a handful of case reports of ET in animals, primarily affecting dogs^{3,14,16–18,25,45,55} and cats.^{17,24} However, after careful scrutiny, many of these cases were probably not ET (as currently defined in humans) and were more likely reactive thrombocytosis,^{16–18} acute megakaryoblastic leukemia⁵⁵ or CML.^{14,16,25,45} In the two cases of what may be true ET (in a dog and a cat), PVSG criteria (Table 80.3) were used for diagnosis.^{3,24} Both animals had a marked persistent thrombocytosis (>600,000 platelets/ μ L for >1 month), megakaryocytic hyperplasia, normal TPO levels, and no evidence of dysplasia, iron deficiency, or underlying inflammation. It is possible that ET is a different disorder in animals than in humans and diagnostic criteria for humans (including genetic mutations) may not be valid in animals.

The main differential diagnosis for ET in animals is a reactive or cytokine-driven thrombocytosis, which is

far more common (regardless of the platelet count). Essential thrombocythemia is an extremely rare diagnosis in animals and, as in humans, remains one of exclusion. It is very difficult to differentiate ET from other CMPDs (e.g. CML), which are also quite rare. A diagnosis of ET should only be entertained in animals with a sustained (more than 1–2 months) and unexplained thrombocytosis and no evidence of CML (low numbers of myeloblasts, and no dysplasia, leukocytosis or severe anemia). It is possible that the increased availability of sensitive markers of inflammation (e.g. acute phase reactant proteins⁹) will help distinguish between reactive thrombocytosis and ET in animals.

Thrombocytosis has also been reported with acute megakaryoblastic leukemia^{7,10,44} and other CMPDs, including basophilic leukemia and PV in animals.^{36,51}

Familial/Inherited Thrombocytosis

This has been attributed to germline mutations in genes involved in thrombopoiesis (MPL, TPO, JAK2), resulting in non-neoplastic polyclonal thrombopoiesis. High platelet counts are observed at birth or an early age. Affected individuals may be asymptomatic or suffer from thrombohemorrhagic complications.^{4,52,53}

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Von Willebrand Disease

MARJORY B. BROOKS and JAMES L. CATALFAMO

Disease Mechanism	Functional and structural VWF Assays
Disease Classification	Point of Care Analyses
VWD Subtypes	Clinical Management of VWD
Affected species and breeds	Clinical Diagnosis
Inheritance and Expression Patterns	Clinical Signs
Hereditary VWD	Diagnostic Evaluation
Acquired VWD	Treatment of VWD
von Willebrand Factor Assays	Transfusion Therapy
Quantitative VWF Assays	Non-transfusion Therapy

Acronyms and Abbreviations

AVWS, acquired von Willebrand syndrome; ADAMTS13, a disintegrin and metalloprotease with thrombospondin repeats; DDAVP, deamino 8-D-arginine vasopressin; ELISA, enzyme-linked immunosorbent assay; FVIII, coagulation factor VIII; LIA, latex immunoassay; OMIM, on-line inheritance in man; PFA, platelet function analyzer; MW, molecular weight; VWD, von Willebrand disease; VWF, von Willebrand factor; VWF:Ag, von Willebrand factor antigen; VWF:CB, von Willebrand factor collagen binding; VWF:RCo, von Willebrand factor ristocetin cofactor activity.

Von Willebrand disease (VWD) is the most common hereditary bleeding disorder in dogs¹⁰ and people,²¹ and the trait has been reported in many other species. The disease is heterogeneous, caused by a variety of defects in von Willebrand factor protein (VWF) that influence clinical severity and complicate disease diagnosis and management.^{6,9,10,19–21}

DISEASE MECHANISM

The bleeding tendency of VWD is caused by quantitative and functional deficiencies of VWF, a large plasma glycoprotein required for platelet adhesion at sites of vessel injury (Fig. 81.1).²¹

Endothelial cells are the major site of VWF synthesis and storage. Platelets provide a secondary pool of VWF in some species; however, canine platelets contain only trace amounts of VWF. Mature VWF circulates as linear strings of subunits (multimers) assembled within the endoplasmic reticulum and Golgi apparatus. Initially, two VWF subunits join to form dimers, followed by the association of dimers into variably-sized multimers.

Multimers may be composed of more than 100 subunits, ranging in molecular weight (MW) from 500 to 20,000 kDa. Endothelial cells secrete VWF via steady-state constitutive pathways, and release the protein from storage organelles in response to stimuli such as thrombin and epinephrine. VWF acts as a carrier for coagulation factor VIII (FVIII), circulating with FVIII in a noncovalent complex. Upon vascular injury, VWF binds to subendothelial collagen and undergoes a conformation change that facilitates its interaction with platelet glycoprotein Ib. VWF also mediates intraplatelet bridging via platelet glycoprotein IIb/IIIa. High MW VWF multimers are most effective in supporting platelet adhesion. Deficiency of VWF, or loss of high MW forms, results in failure of platelet plug assembly, especially under high shear in the microvasculature. After secretion, ultra-high MW multimers are catabolized to smaller forms by a disintegrin and metalloprotease with thrombospondin repeats (ADAMTS13) (Fig. 81.1).²¹ This conversion takes place slowly in plasma under static conditions (12 hour half-life); however, ADAMTS13 more rapidly cleaves platelet-bound VWF under conditions of high shear.^{5,21}

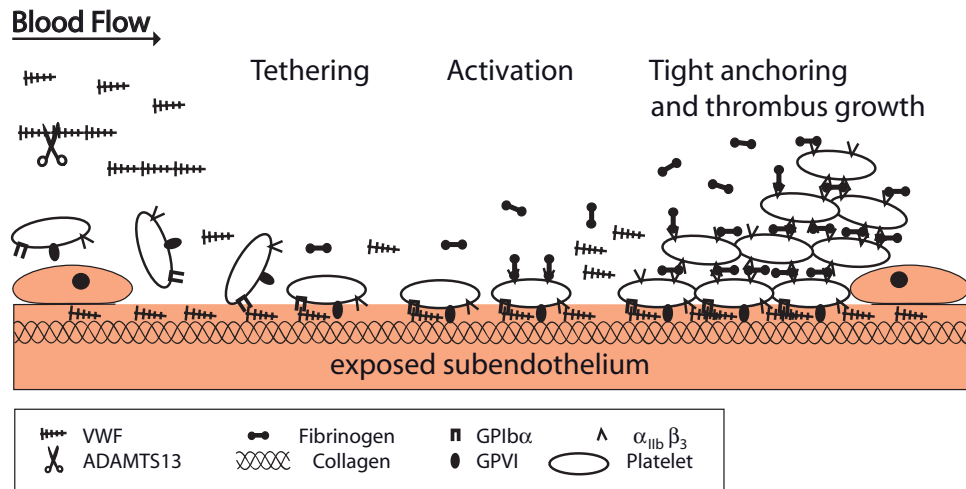


FIGURE 81.1 Schematic diagram illustrating the role of VWF in platelet adhesion, aggregation, and thrombus growth. At the site of blood vessel injury, platelets transiently adhere to subendothelial VWF via platelet GPIb α receptors. This slows platelet movement and triggers the engagement of platelet receptors, including the collagen receptor. Receptor engagement results in platelet activation and stable attachment. Activated platelets secrete additional prostimulatory molecules and bind to plasma fibrinogen and VWF to form a tight anchoring matrix for platelet recruitment and ultimately thrombus formation. Upon release from endothelial cells, ultra large VWF multimers are rapidly cleaved by ADAMTS13.

TABLE 81.1 Classification of von Willebrand Disease

Type	VWF Defect	Affected Species (Breeds)
1	Partial quantitative deficiency, residual VWF has normal structure and function	Dog (Airedale, Akita, Bernese mountain dog, Dachshund, Doberman pinscher, German shepherd, Golden retriever, Greyhound, Irish wolfhound, Kerry blue terrier, Manchester terrier, Miniature pinscher, Papillon, Pembroke Welsh Corgi, Poodles, Schnauzer, other purebreeds and mixed breed dogs) Horse (Arabian) Mouse (RIIS/J)
2A	Selective loss of large VWF multimers, decreased VWF-platelet & collagen interactions	Dog (German shorthaired pointer, German wirehaired pointer) Cow (Simmental) Horse (Quarter Horse, Thoroughbred)
2B	Increased VWF affinity for platelet glycoprotein Ib	No animal cases
2M	Impaired VWF binding to platelet glycoprotein Ib, normal multimer structure	No animal cases
2N	Decreased VWF binding to factor VIII	No animal cases
3	Complete VWF deficiency	Dog (Dutch Kooiker, Scottish terrier, Shetland sheepdog; sporadic cases Border collie, Chesapeake Bay retriever, Cocker spaniel, Eskimo dog, Labrador retriever, Maltese, Pitbull, and mixed breed) Cat (Himalayan) Pig (Poland, China) Primate (Rhesus monkey)

DISEASE CLASSIFICATION

VWD Subtypes

Von Willebrand disease in people is classified into one of three categories based on plasma VWF concentration, function, and multimer structure (see Table 81.1).^{6,21} This classification scheme is broadly applicable for VWD in animals. Type 1 VWD is a partial quantitative deficiency. Plasma VWF concentration is low (<50%

of normal), but the protein consists of a full complement of functional multimers. In this form, clinical severity generally correlates with reduction in VWF concentration.^{3,19,21}

Type 2 VWD refers to a group of VWF functional variants. Although four distinct subtypes are found in people, type 2A VWD is the only subtype described in animals to date.^{2,16,24} In this form, preferential loss of high MW multimers accompanies low plasma VWF concentration. The residual VWF’s ability to support

platelet adhesion is greatly reduced. Defects that enhance the binding affinity of the platelet VWF receptor (glycoprotein 1b α) produce a disease phenotype similar to type 2B VWD. This rare bleeding disorder, referred to as platelet-type VWD, has not been identified in animals.

Type 3 VWD refers to a complete absence of plasma VWF. Lack of VWF, in many species, is associated with concomitant reduction in FVIII. However, dogs affected with type 3 VWD have at most mild reduction in FVIII, presumably due to species-differences in FVIII stability.²²

Affected Species and Breeds

Clinical expression of VWD is best characterized in dogs;^{1-3,10,12,17,26} however, family studies and case reports describe VWD in horses,¹⁶ non-human primates,¹⁵ cats,⁷ cows,²⁴ and laboratory strains of pigs⁸ and mice¹³ (Table 81.1). Type 1 VWD is the most common form in dogs, with cases identified in many purebreds and in mixed breed dogs (Table 81.1). Type 2 VWD represents 10–20% of human cases,^{19,21} however, this subtype has been found only in two related canine breeds (German wirehaired and shorthaired pointers) in addition to horses and cattle. The most severe form, type 3 VWD, has been described as a familial trait in several breeds (Kooiker, Scottish terrier, Shetland sheepdog), and there are many sporadic cases in other breeds and species (Table 81.1). The true prevalence of VWD within affected breeds is difficult to determine. In the absence of random surveys, diagnosis bias favors screening affected individuals and their relatives. Selection may eliminate the trait within a certain family, in spite of relatively high overall breed prevalence. The breed-variants listed in Table 81.1 include published reports and recent case submissions to the authors' laboratory; however, new cases may develop in previously unaffected species and breeds.

INHERITANCE AND EXPRESSION PATTERNS

Molecular characterization of VWD reveals a variety of mutation-types and numerous distinct mutations throughout the VWF gene (*VWF*).^{9,17,21,26} In general, mutations causative for type 2 VWD are clustered in *VWF* regions required for multimer assembly and stability, or VWF-platelet interactions. Common mutations in type 3 patients include frameshift, nonsense, and large deletions that disrupt VWF synthesis. Mutations causative for type 1 VWD are found throughout *VWF* and in some variants, described in mice and people, mutations unlinked to the *VWF* locus influence VWF protein stability or rate of synthesis.^{9,13,21}

Hereditary VWD

Hereditary VWD is an autosomal trait. In this form of inheritance, males and females transmit and express the trait with equal frequency. Most type 2 VWD kindreds

TABLE 81.2 Transmission and Expression of Recessive von Willebrand Disease

Parental Status	Offspring Status: Predicted Ratios ^a
Clear (VV) \times Clear (VV)	All Clear (VV)
Clear (VV) \times Carrier (VV ^m)	½ Clear (VV) & ½ Carrier (VV ^m)
Carrier (VV ^m) \times Carrier (VV ^m)	¼ Clear (VV) & ½ Carrier (VV ^m) & ¼ Affected (V ^m V ^m)
Clear (VV) \times Affected (V ^m V ^m)	All Carrier (VV ^m)
Carrier (VV ^m) \times Affected (V ^m V ^m)	½ Carrier (VV ^m) & ½ Affected (V ^m V ^m)
Affected (V ^m V ^m) \times Affected (V ^m V ^m)	All Affected (V ^m V ^m)

^a(VV) = VWD Clear, male or female, no signs of bleeding; (VV^m) = VWD Carrier, male or female, no signs of bleeding; (V^mV^m) = VWD Affected, male or female, clinical bleeding due to abnormal primary hemostasis.

and all type 3 VWD families demonstrate recessive expression patterns. Homozygotes inherit mutant *VWF* alleles from both parents and invariably express a bleeding tendency, whereas heterozygotes have one normal and one mutant *VWF* allele and are clinically normal (see Table 81.2).

Two distinct mutations have been described in canine breed-variants of type 3 VWD.^{17,26} A deletion mutation within exon 4 and a splice site mutation at the boundary of exon 16 are causative for type 3 VWD in Scottish terriers and Dutch Kooikers, respectively. Direct DNA tests are commercially available (Vetgen) to detect these mutations, and other mutations believed to be causative for breed-variants of type 2 or type 3 (Shetland sheepdog) VWD. Heterozygous carriers of types 2 and 3 VWD have low plasma VWF concentration, with values generally less than 50% of normal. Carrier status may be ambiguous for some individuals, however, due to overlap in VWF values at the low end of normal range.^{2,9,10}

Type 1 VWD is typically a dominant trait, or dominant with incomplete penetrance.^{9,19,20} The causative mutations underlying this phenotype in people are often classified as "dominant-negative," where an abnormal gene product affects VWF processing or clearance. Incomplete penetrance refers to a variability of disease expression among individuals carrying the same mutation. A range of clinical severity is also a common feature within canine breed-variants of type 1 VWD.^{3,10,18} While low plasma VWF concentration is a primary risk factor for expression of type 1 VWD, molecular genetic and biochemical analyses have not fully defined additional factors that influence disease penetrance.^{9,13,21} A commercial test (Vetgen) is offered to detect a *VWF* splice site mutation common to many canine breeds affected with type 1 VWD. The relationship of homozygosity or heterozygosity for this mutation to expression of a bleeding tendency has not been described; however, association studies in types 1 and 2 VWD have shown that the canine *VWF* locus has a major influence on plasma VWF concentration.^{3,12}

Acquired VWD

Acquired von Willebrand syndrome (AVWS) refers to VWF quantitative and functional defects that develop as a consequence of other primary disease conditions.²¹ The pathogenesis of AVWS in people includes antibody-mediated clearance secondary to immune disorders, shear-induced proteolysis due to cardiac disease, and increased VWF-platelet binding in thrombotic and neoplastic syndromes. In addition to these defined mechanisms, AVWS has been described in people (and animals) in association with hypothyroidism and treatment with plasma expanders (e.g. hydroxyethyl starch, dextran). An AVWS, characterized by loss of high MW VWF multimers, has been reported recently in dogs with cardiac valvular lesions causing turbulent flow.²⁵ Screening to detect VWF defects is therefore warranted in animals with signs of mucosal hemorrhage and primary diseases associated with AVWS. Similarly, correction of acquired disorders that reduce VWF plasma half-life or impair its function may prevent exacerbation of hereditary VWD.

VON WILLEBRAND FACTOR ASSAYS

Definitive diagnosis of VWD is based on specific assay of plasma VWF.⁶ Species differences in VWF antigenic structure and function require species-specific assays, or validation of human assays, for veterinary applications. Valid results also depend on collection techniques that prevent platelet activation and clot formation, conditions that deplete and degrade VWF in the sample specimen.

Quantitative VWF Assays

VWF antigen (VWF:Ag) refers to the immunologic quantitation of VWF concentration. Determination of VWF:Ag is the initial step in characterizing VWD. The most sensitive and accurate assay methods in current use are enzyme-linked immunosorbent assay (ELISA) and automated latex immunoassay (LIA).⁶ Test results of VWF (and clotting factors) are conventionally reported as percentage of “normal.” The VWF content of each test sample is compared with that of a reference standard having an assigned value of 100% or 100 units per deciliter (U/dL). Assay standards and reference intervals for animal VWF:Ag must be established by each testing laboratory. In general, plasma VWF:Ag below 50% (<50 U/dL) indicates VWF deficiency.

Functional and Structural VWF Assays

Functional VWF assays measure various aspects of VWF interaction with platelets, collagen, or FVIII. The ristocetin cofactor assay (VWF:RCo) is a traditional test that measures VWF-dependent platelet agglutination. This property of VWF depends on the presence of high MW multimers and that VWF:RCo values are disproportionately decreased compared to VWF:Ag in people

with type 2A, 2B, and 2M VWD.^{6,21} VWF:RCo assays are difficult to standardize for clinical diagnosis in people and require modification for use in animals. Collagen binding assays (VWF:CB) measure a different function of VWF that also depends on the presence of high MW VWF multimers. VWF:CB assays have begun to replace VWF:RCo for initial screening to detect type 2 VWD. These newer assays are more readily standardized among laboratories, and have been adapted for clinical assessment of VWD subtypes in dogs.^{11,20}

VWF multimers are visualized using protein electrophoresis for size separation, followed by direct hybridization with anti-VWF probes, or transfer to a membrane (western blot) before antibody labeling. Multimer analyses are generally performed in research settings for detailed VWF characterization and VWD subtype classification of newly diagnosed breed or species-variants.^{2,4,10}

Point-of-Care Analyses

Bleeding time tests are *in vivo* measures of primary hemostasis performed by making a superficial incision in a capillary bed and monitoring the time to cessation of blood flow. Prolongation of bleeding time is compatible with severe forms of VWD (and platelet function defects); however, the bleeding time endpoint is also influenced by platelet count, hematocrit, and other conditions affecting blood viscosity.⁶ In addition to nonspecificity, bleeding time tests have fallen out of use in human medicine because of high inter-operator variability, patient discomfort, and poor predictive values.

The Platelet Function Analyzer (PFA-100®, Dade Behring) is a table-top instrument that measures platelet plug formation in whole blood under high shear flow.^{6,14} The test endpoint (closure time) refers to occlusion of an aperture coated with collagen and ADP or epinephrine. The instrument was developed as a non-invasive test of platelet and VWF function and has been evaluated in dogs and horses. The PFA-100® ADP/collagen closure time is sensitive to VWF deficiency and dysfunction in dogs, with reports of prolonged closure times in dogs with types 1, 2, and 3 VWD.^{4,14} Many of the nonspecific conditions that influence bleeding time (e.g. thrombocytopenia, anemia) also affect the closure time endpoint, thereby limiting its use as a screening test in some clinical situations.

CLINICAL MANAGEMENT OF VWD

Clinical Diagnosis

Clinical Signs

Clinical signs of VWD in all species include mucosal hemorrhage, cutaneous bruising, and prolonged bleeding after surgery or trauma. Specific sites of mucosal hemorrhage in VWD case series include epistaxis, hematuria, gastrointestinal hemorrhage, prolonged

estral bleeding, and gingival bleeding at deciduous teeth.¹ These signs are typical of primary hemostatic defects, commonly seen in patients with thrombocytopenia, platelet dysfunction, and VWD. Petechiae, however, represent specific signs of platelet defects and are rarely seen in VWD patients. The bleeding tendency of type 1 VWD ranges from mild to severe. Mildly affected patients infrequently develop spontaneous hemorrhage, but typically experience abnormal bleeding after surgery or trauma. Types 2 and 3 VWD are invariably severe bleeding disorders. Neonatal deaths caused by hemorrhage may occur, but many animals survive to adulthood, albeit with transfusion support. The anatomic site of tissue injury influences the consequences of hemorrhage. Minor hemorrhage into critical sites such as the central nervous system or respiratory tract may produce severe morbidity and mortality. Oronasal and urinary tract tissues are rich in fibrinolysins, and injuries to these tissues often cause more prolonged bleeding than superficial wounds.

Diagnostic Evaluation

Thrombocytopenia and coagulation factor deficiencies are common causes of hemorrhage; therefore the initial evaluation of any patient suspected of having a bleeding diathesis should include platelet count and coagulation screening tests (see Fig. 81.2).

Bleeding scores derived from standardized questionnaires have been proposed to improve the diagnostic utility of VWD screening in people.¹⁹ A brief hemostasis questionnaire is also useful as a screening tool to identify veterinary patients with hereditary or drug-induced bleeding disorders (see Table 81.3). If preliminary results reveal normal platelet count and coagulation panel results, then point-of-care tests of primary hemostasis and/or VWF:Ag should be performed to screen for VWD (see Fig. 81.2). Prolongation of in vivo bleeding time (beyond 4–6 minutes) and PFA-100® collagen/ADP closure time (beyond 2 minutes) is compatible

TABLE 81.3 Screening Questionnaire to Aid in the Diagnosis of Bleeding Disorders

- 1 Has this animal ever had spontaneous bleeding from nose, mouth, urinary tract, or noticeable bleeding at teething?
- 2 Has this animal ever had a surgical procedure, dentistry, or traumatic injury – did bleeding complications occur?
- 3 Has this animal ever required a transfusion because of anemia or hemorrhage?
- 4 Is this animal receiving medication or dietary supplements?
- 5 Are you aware of any bleeding problems in relatives or in this breed?

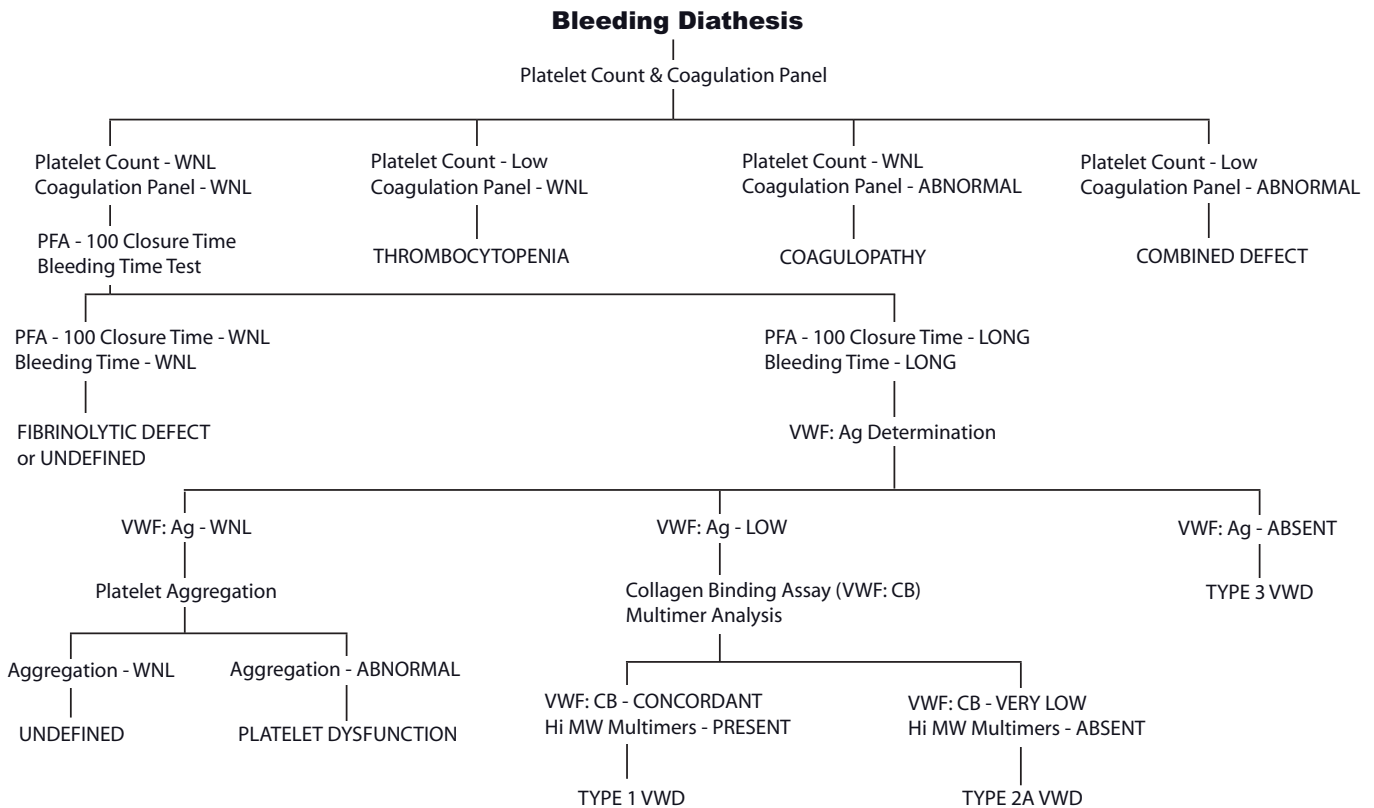


FIGURE 81.2 Diagnostic algorithm for assessment of VWD and other bleeding disorders.

with VWD, and the finding of low plasma VWF (VWF:Ag < 50%) confirms VWF deficiency. A complete lack of plasma VWF (VWF:Ag < 0.1%) is definitive for severe, type 3 VWD. Diagnosis of type 2 VWD is based on quantitative deficiency, combined with additional structural and functional abnormalities (see Fig. 81.2 and Table 81.1). In particular, a disproportionate defect of collagen binding (ratio of VWF:Ag/VWF:CB > 2) provides evidence of type 2A VWD. In patients with low, but functional VWF, the risk of abnormal hemorrhage ranges along the continuum of VWF:Ag values. In dogs and people, clinical expression of a bleeding tendency is typically associated with VWF:Ag < 25%, whereas VWF:Ag close to 50% is often clinically inapparent.^{1,3,9,19} The current management strategy for type 1 VWD in people considers VWF deficiency as a biomarker for hemostatic risk. Ongoing studies aim to relate clinical outcomes to defined VWF protein levels, mutation types, and other parameters of intrinsic platelet function.^{9,21}

Treatment of VWD

Management of VWD is directed at controlling hemorrhage in patients with active bleeds and preventing hemorrhagic complications in case of surgery or trauma. The clinical heterogeneity of VWD requires an individualized approach to account for disease classification and underlying conditions that might influence disease expression.

Transfusion Therapy

Transfusion is the mainstay of emergency treatment and prophylaxis of VWD (see Chapter 90 and Section VIII). The most successful strategy is early, high-dose transfusion to rapidly increase plasma VWF to levels capable of supporting platelet adhesion. Patients with types 2 and 3 VWD almost invariably require transfusion to undergo invasive procedures or stop an active bleed. Transfusion for type 1 VWD can be assessed based on clinical history, severity of VWF deficiency, any underlying disease, and the nature of the hemostatic stress. Patients with blood-loss anemia or requiring major surgical procedures should be transfused, whereas those with normal hematocrits or minor, cutaneous injuries may be successfully managed with limited or no transfusion. Severely affected patients may require repeated transfusion, with lower dosages at longer intervals to sustain hemostasis for 24–48 hours after initial control of bleeding. Stabilization of hematocrit and cessation of active hemorrhage are signs that hemostatic levels of VWF have been attained.

Plasma cryoprecipitate is the most specific component for rapid VWF replacement therapy, with fresh frozen plasma an acceptable alternative (see Chapter 96).²³ The use of plasma components, rather than whole blood, prevents sensitization to red cell antigens, eliminates the need for type-matched donors, and minimizes risk of volume overload. The unit definition of cryopre-

cipitate varies for different suppliers; however, transfusion of 1 unit/10 kg cryoprecipitate (1 unit defined as cryoprecipitate from 200 mL fresh frozen plasma) or transfusion of 10–15 mL/kg fresh frozen plasma provides adequate VWF to support hemostasis. The plasma half-life of VWF is approximately 12 hours, necessitating q8–12 hour transfusion for severe hemostatic stress.

Non-transfusion Therapy

Hereditary VWD causes a life-long bleeding tendency; therefore invasive procedures are best avoided. In case of injury or non-elective surgery, local wound care may help reduce transfusion requirements (see Chapter 90). Cautery, topical tissue adhesive, multilayer closure, and pressure wraps help to reduce blood loss at focal injury sites. Anti-platelet and anticoagulant drugs (e.g. sulfa drugs, non-steroidal anti-inflammatory agents, heparin, dextran, and other plasma expanders) should be given cautiously, if at all.

Signs of VWD can be exacerbated by concurrent thrombocytopenia and other disease conditions that affect hemostasis. Uremia, hyperproteinemia, anemia, hypothyroidism, and liver disease are among the more common disease conditions associated with primary hemostatic failure. Medical therapy to correct these disorders, and primary diseases associated with AVWS, is therefore important in the general management of VWD.

Desmopressin acetate (deamino 8-d-arginine vasopressin [DDAVP]) is a synthetic vasopressin analog used as a specific pharmacologic agent for type 1 VWD in people. The drug is believed to act by stimulating endothelial V₂ vasopressin receptors, resulting in release of VWF intracellular stores. Repeated infusions of desmopressin at closely spaced intervals fail to elicit VWF release (tachyphylaxis), thereby limiting its ability to maintain hemostasis to periods of hours, rather than days. Desmopressin has been recommended as pre-operative prophylaxis for type 1 VWD-affected dogs, although experimental studies reveal relatively small increases in plasma VWF concentration compared to the human response.^{4,11} To support surgical hemostasis, desmopressin (1 µg/kg) is given subcutaneously one-half hour before surgery. Close monitoring is required, with transfusion available if desmopressin response is inadequate.

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ON-LINE RESOURCES

1. **Comparative Coagulation Laboratory.** Animal von Willebrand factor assays. <http://www.diaglab.vet.cornell.edu/coag/>
2. **NIH.** Guidelines on diagnosis and management of VWD. <http://www.nhlbi.nih.gov/guidelines/vwd/vwd.pdf>
3. **OMIM.** Comprehensive summary and links to molecular and biochemical characterization of VWF and VWD. <http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=193400>
4. **VetGen.** Mutation detection tests for VWD in dogs. <http://www.vetgen.com/>

Inherited Intrinsic Platelet Disorders

MARY K. BOUDREAU

Overview – Intrinsic vs Extrinsic Platelet Disorders
 Inherited Macrothrombocytopenia
 Membrane Receptor Disorders
 Glanzmann Thrombasthenia (GT)
 P2Y₁₂ Receptor Disorder
 Storage Pool Disorders
 Cyclic Hematopoiesis
 Chediak-Higashi Syndrome (CHS)
 Dense Granule Defect

Signal Transduction Disorders
 Calcium Diacylglycerol Guanine Nucleotide
 Exchange Factor I (CalDAG-GEFI) Thrombopathias
 Kindlin-3
 Procoagulant Expression Disorders
 Scott Syndrome
 Diagnosis
 Case Management

Acronyms and Abbreviations

ADAMTS13, a disintegrin and metalloprotease with thrombospondin repeats; AP3 β 1, adaptor protein complex 3 beta 1; CalDAG-GEFI, calcium diacylglycerol guanine nucleotide exchange factor I; CAP-1, canine activated platelet-1; CHS, Chediak-Higashi Syndrome; CKCS, Cavalier King Charles Spaniel; DDAVP, 1-desamino-8-D-arginine vasopressin; guanine nucleotide exchange factor (GEF GT, Glanzmann thrombasthenia; RIBS, receptor-induced binding site; VWD, von Willebrand Disease.

OVERVIEW – INTRINSIC VS EXTRINSIC PLATELET DISORDERS

Intrinsic platelet disorders are characterized by defects involving membrane receptor glycoproteins, secretory organelles, signal transduction proteins, or structural proteins that result in either platelet dysfunction (thrombopathia) or thrombocytopenia. Several inherited intrinsic platelet disorders have been documented in veterinary medicine at the functional, biochemical, and molecular level. Extrinsic platelet disorders are characterized by abnormalities in plasma or subendothelial proteins that are essential for platelet adhesion or aggregation. Extrinsic platelet disorders include von Willebrand Disease (VWD) and hypo- or dysfibrinogenemia, the latter of which are rare in veterinary medicine. Intrinsic and extrinsic platelet disorders are indistinguishable at the clinical level. Buccal mucosa bleeding times are usually prolonged in both types of disorders while coagulation screening tests are normal. Petechial and ecchymotic hemorrhages and mucosal bleeding such as gingival bleeding, epistaxis, and urinary and gastrointestinal hemorrhage can be observed with both types of disorders. Internal bleeding within organs such as kidney, spinal cord, and brain can also occur. Excessive bleeding during permanent tooth eruption is commonly observed and may be the

first indication of the existence of a bleeding diathesis. Clinical signs in affected animals can vary considerably; signs may be overt or very subtle or nonspecific. Periodic bruising or petechial hemorrhages may be the only clinical signs. Internal hemorrhage within the brain or spinal cord may result in seizures or paralysis. Unilateral or bilateral epistaxis occurs in some affected animals and may be either spontaneous or associated with treatment with platelet-inhibitory medications. Trauma or surgery can result in severe hemorrhage with either intrinsic or extrinsic platelet disorders. Transfusion products required for treatment of intrinsic and extrinsic platelet disorders differ; therefore correct classification is necessary prior to development of an appropriate treatment regimen for a bleeding patient.

INHERITED MACROTHROMBOCYTOPENIA

Cavalier King Charles Spaniels (CKCS) have an autosomally recessive inherited macrothrombocytopenia that is not associated with clinical bleeding.^{21,25,41,46,48} Platelet counts can range between 30,000 and 100,000/ μ L and large platelets are frequently observed. Platelets in affected CKCS have been reported as being both hypo- and hyper-responsive; discrepancies are likely due to variations in methodology and in the CKCS population

TABLE 82.1 Genetic Basis for Inherited Intrinsic Platelet Disorders Reported in Dogs, Cattle, and Horses

Disorder/Breed	Gene ^a	Mutation ^b
Macrothrombocytopenia		
Cavalier King Charles Spaniel	<i>β1-Tubulin</i>	Exon 4, G>A, D>N
Glanzmann thrombasthenia		
Great Pyrenees	<i>αIIb</i>	Exon 13, 14-base duplication
Otterhound	<i>αIIb</i>	Exon 12, G>C, D>H
Thoroughbred (England)	<i>αIIb</i>	Exon 2, G>C, R>P
Quarter Horse (Alabama) ^c	<i>αIIb</i>	Exon/Intron 11, 10-base deletion; Exon 2, G>C, R>P
Peruvian Paso (Idaho)	<i>αIIb</i>	Exon/Intron 11, 10-base deletion
Oldenbourg (Canada)	<i>αIIb</i>	Exon 2, G>C, R>P
Cyclic hematopoiesis		
Gray collie	<i>AP3β1</i>	Exon 20, 1-base duplication
ADP Receptor		
Greater Swiss Mountain Dog	<i>P2Y12</i>	Exon 2, 3-base deletion
Chediak-Higashi		
Japanese Black cattle	<i>LYST/CHS1</i>	6065A>G, H>R
Selective ADP deficiency		
American Cocker Spaniel		Not known
Signal transduction		
Basset hound	<i>CaIDAG-GEFI</i>	Exon 5, 3-base deletion
Spitz	<i>CaIDAG-GEFI</i>	Exon 5, 1-base duplication
Landseer-ECT	<i>CaIDAG-GEFI</i>	Exon 8, C>T, R>STOP
Simmental cattle	<i>CaIDAG-GEFI</i>	Exon 7, T>C, L>P
Arabian horse	<i>CaIDAG-GEFI</i>	Exon 6, C>G, H>Q
German Shepherd	<i>Kindlin-3</i>	Exon 12, 12-base insertion
Procoagulant expression		
German Shepherd		Not known

^a*CaIDAG-GEFI*, calcium diacylglycerol guanine nucleotide exchange factor I.

^bD, Aspartic acid; H, histidine; R, arginine; L, leucine; P, proline; N, asparagine; Q, glutamine.

^cCompound heterozygote.

evaluated. The prolonged PFA-100 closure times reported in some CKCS are likely secondary to acquired VWD in dogs with concomitant mitral valve regurgitation and are not related to the inherited macrothrombocytopenia.⁵¹ The high shear conditions generated with mitral valve regurgitation and other types of heart valve disorders can result in enhanced cleavage of von Willebrand Factor (VWF) by ADAMTS13 (a disintegrin and metalloprotease with thrombospondin repeats), an enzyme that cleaves VWF under high shear conditions.⁵² Evaluation of plasma VWF antigen concentration and VWF collagen binding activity should be included in the diagnostic profile. The large platelets in affected CKCS can be equal in size or larger than erythrocytes and tend to be spherical to oval in shape with prominent alpha granule staining. A mis-sense mutation at coding nucleotide 745 (c.745G>A) in the gene encoding β1-tubulin correlates with the inherited macrothrombocytopenia in CKCS.²² The mutation is predicted to result in the substitution of an asparagine for aspartic acid at amino acid position 249 (D249N) (Tables 82.1 and 82.2). Aspartic acid 249 is part of a microtubule intraprotofilament binding site and the change in charge at this position is thought to impair microtubule assembly necessary for normal proplatelet formation and platelet production by megakaryocytes. The prevalence of the mutation in United States CKCS is high; in the study by Davis et al., 90% of the CKCS were found to be either homozygous or heterozygous for the muta-

tion. In the same study 65% of CKCS in Ireland were either heterozygous or homozygous for the mutation. Treatment is not necessary for this disorder; affected dogs do not have clinical signs.

Recently two non-CKCS dogs were documented to be heterozygous for the β1-tubulin gene mutation described in CKCS (M.K. Boudreaux, personal observation). Mutations in β1-tubulin should be considered as part of the differential diagnosis in dogs with persistent thrombocytopenia in the absence of clinical signs.

MEMBRANE RECEPTOR DISORDERS

Glanzmann Thrombasthenia (GT)

Glanzmann thrombasthenia is characterized by absence or marked reduction in platelet glycoprotein complex IIb-IIIa, also known as integrin αIIb-β3 and as the fibrinogen receptor.³⁹ In people, GT is categorized into three main types: Type I, Type II, and Variant. In Type I less than 5% of the receptor is detectable on platelet surfaces and clot retraction is absent. In Type II 10–20% of the receptor is present on platelet surfaces and clot retraction is detectable but reduced. In Variant GT the receptor is present but dysfunctional; clot retraction may or may not be detectable. In veterinary medicine, Type I GT has been documented in horses and dogs.^{5,24,33,36} The αIIb-β3 complex primarily binds fibrin-

TABLE 82.2 Comprehensive Description of Reported Mutations Causing Animal Platelet Disorders

Macrothrombocytopenia Cavalier King Charles Spaniel	There is a substitution of an A for a G in the gene encoding β 1-tubulin at coding nucleotide 745 (c.745G>A) resulting in the substitution of an asparagine for aspartic acid at amino acid 249 (D249N). Microtubule stability is impaired resulting in aberrant proplatelet formation and platelet release. Affected dogs do not have clinical signs
Glanzmann thrombasthenia Great Pyrenees	A 14-base duplication near the end of exon 13 of the gene segment encoding for the fourth calcium binding domain of α IIb results in defective splicing of intron 13 and appearance of a premature stop codon
Otterhound	A single nucleotide change at coding nucleotide 1100 (c.1100G>C) in exon 12 of the gene encoding for α IIb results in the substitution of a histidine for an aspartic acid in the third calcium binding domain of α IIb (D367H). This results in a charge change in a highly conserved region of the protein. Similar mutations in humans resulted in destabilization of the α IIb- β 3 complex resulting in lack of expression of the complex on the platelet surface
Thoroughbred-cross (England) and Oldenbourg (Canada) Quarter Horse (Alabama)	A single nucleotide change at coding nucleotide 122 (c.122G>C) in exon 2 of the gene encoding α IIb results in the substitution of a proline for an arginine (R41P) in a highly conserved region of the protein
Peruvian Paso (Idaho)	This horse is a compound heterozygote with the same mutation described in the Thoroughbred in England as well as a 10-base deletion encompassing the splice site at the end of exon 11 in the gene encoding for α IIb. This second mutation likely results in lack of synthesis of the protein due to the appearance of a premature stop codon leading to nonsense-mediated decay
Cyclic hematopoiesis Gray collie	This horse is homozygous for the 10-base deletion in the gene encoding α IIb described in the Quarter horse in Alabama
Chediak-Higashi syndrome Japanese Black cattle	There is a 1-base duplication (A) in a tract of 9 adenine nucleotides in exon 20 of the gene encoding the beta subunit of adaptor protein complex 3. The mutation and resulting frameshift cause the appearance of a premature stop codon. The protein is part of a complex that directs trans-Golgi export of transmembrane cargo proteins to granules
CalDAG-GEFI disorder Basset hounds	There is a single nucleotide change (c.6065A>G) resulting in the substitution of an arginine for a histidine (H2015R) in the gene encoding a lysosomal trafficking regulator protein referred to as LYST or CHS1
Eskimo Spitz	There is a deletion in coding nucleotides 509, 510, and 511 (c.509–511delTCT) of the gene encoding CalDAG-GEFI resulting in deletion of a phenylalanine at position 170 (p.F170del) in structurally conserved region 1 (SCR1) of the catalytic domain of the protein. This mutation results in a qualitative disorder; the protein can be detected in western blots
Landseers-ECT	There is a duplication of coding nucleotides 452 (c.452dupA) of the gene encoding CalDAG-GEFI resulting in a frame shift starting at the codon encoding aspartic acid 151 (D151) near the beginning of SCR1. This frame shift results in the appearance of a premature stop codon at nucleotide 796.
Simmental cattle	There is a premature stop codon at the codon encoding arginine at position 328 (R328Stop) of the gene encoding CalDAG-GEFI due to a substitution at coding nucleotide 982 (c.982C>T) at the beginning of the sequence encoding SCR4. The protein is not synthesized likely due to nonsense mediated decay of mRNA.
Arabian horse	There is a substitution at coding nucleotide position 701 (c.701T>C) in the gene encoding CalDAG-GEFI resulting in the change of a leucine to a proline in SCR2 at amino acid 234 (L234P).
	There is a single nucleotide change at coding nucleotide 687 (c.687C>G) predicted to result in the change of a histidine to a glutamine at position 229 (H229Q) in SCR2. Although this mutation is within a SCR, the platelet function phenotype is mild compared to CalDAG-GEFI disorders described in dogs and cattle.

ogen; however, it can also bind other RGD-containing adhesive proteins, including von Willebrand factor. Glanzmann thrombasthenia is inherited as an autosomal recessive trait and is characterized by the inability of platelets to aggregate in response to all agonists, including thrombin. The alpha and beta subunits of the receptor are encoded by separate genes; both subunits are necessary for the formation of a stable complex

on the platelet surface. All cases of GT described in domestic animals have been due to mutations in the gene encoding the α IIb subunit; mutations in the gene encoding β 3 have not yet been identified.^{7,8,17,18,32} Most mutations are in areas of the gene encoding for calcium binding domains within α IIb (Tables 82.1 and 82.2). α IIb subunits are not detected in flow cytometric assays using antibodies specific to the α IIb subunit. Flow

cytometric studies using antibodies to the $\beta 3$ subunit or to the $\alpha \text{IIb}-\beta 3$ complex may detect $\beta 3$ subunits associated with vitronectin receptors ($\alpha \text{v}-\beta 3$) which share the $\beta 3$ subunit and may be increased in these patients.³⁸ Platelets isolated from dogs with GT do not bind CAP-1, a monoclonal antibody specific for a receptor-induced binding site (RIBS) epitope on canine fibrinogen, when activated by strong agonists.⁶

Glanzmann thrombasthenia (formerly known as thrombasthenic thrombopathia) has been reported in Otterhounds and Great Pyrenees dogs, and in horses in England, Australia, Canada, Japan, and the United States. The prevalence of carriers for the mutation in Otterhounds is between 25% and 30% based on genetic testing done at Auburn University. The prevalence of the mutation in Great Pyrenees dogs is not known; only sporadic testing is being conducted by owners and breeders. Great Pyrenees dogs that are carriers for the mutation have been detected in several states, including Mississippi, Alabama, Florida, Missouri, Minnesota, Indiana, Illinois, Oklahoma, and Washington. The horses described in Australia and Japan have not been documented at the molecular level.^{37,50} The Quarter Horse described at Auburn University was a compound heterozygote and had two distinct mutations in the gene encoding αIIb . One mutation was identical to that described in the horse in England. The other mutation was a 10-base deletion encompassing the exon 11–intron 11 splice site (Tables 82.1 and 82.2). Recently, a 10 year-old horse in Idaho with GT was documented to be homozygous for the same 10-base deletion (M.K. Boudreaux, personal observation). The prevalence of mutations causing GT in horses is unknown.

P2Y12 Receptor Disorder

Recently a Greater Swiss Mountain dog in Calgary, Alberta, Canada was identified with a mutation in the gene encoding P2Y12. The dog experienced excessive bleeding after a routine spay. No other clinical signs were noted. (MK Boudreaux, personal observation).

STORAGE POOL DISORDERS

Cyclic Hematopoiesis

Cyclic hematopoiesis is an autosomal recessive disorder described in gray collies characterized by cyclic fluctuations in the number of circulating neutrophils, reticulocytes, and platelets.^{16,23,34} Melanocytes are also affected in this disorder resulting in the gray color of affected animals. A bone marrow stem cell defect results in neutropenic episodes occurring approximately every 12 days. Mortality is high; most puppies die prior to 6 months of age due to fulminating infection. Thrombocytopenia does not occur and platelet numbers fluctuate between 300,000 and 700,000/ μL . Platelet dense granules are absent. Platelet reactivity to several agonists is defective; clot retraction and platelet adhesiveness are impaired. A mutation in the gene encoding

adaptor protein complex 3 beta-subunit (AP3 $\beta 1$) has been linked to this disorder.² AP3 directs trans-Golgi export of transmembrane cargo proteins to granules.

Chediak-Higashi Syndrome (CHS)

Chediak-Higashi syndrome is an autosomal recessive genetic disorder characterized by abnormal leukocyte, melanocyte and platelet granulation. Affected animals have partial oculocutaneous albinism, increased susceptibility to infection, and a bleeding diathesis as a result of impaired platelet function. Platelets of affected animals lack discernable dense granules and are deficient in granule storage pools of adenine nucleotides, serotonin, and divalent cations. Platelet aggregation in response to collagen, which is largely dependent on thromboxane generation and granule release of ADP, is markedly reduced in affected animals.²⁸ The disease was identified in a line of Persian cats: all of the affected animals exhibited a “blue smoke” hair color and pale irises with the development of bilateral nuclear cataracts in several animals as early as 3 months of age.³⁰ Affected cats experienced prolonged bleeding at incision sites and the development of hematomas following venipuncture. Chediak-Higashi syndrome has also been diagnosed in Aleutian mink,⁴⁰ three breeds of cattle,^{1,40,53} blue foxes,⁴⁷ killer whales,⁴⁴ and mice.³⁵ CHS in Japanese Black cattle, mice, and people has been linked to mutations in the lysosomal trafficking regulator (LYST) gene which encodes a 425 kDa cytoplasmic protein that may be involved with incorporation of proteins into lysosomal membranes.³¹ Molecular studies have not been reported in Brangus or Hereford cattle or other species affected with CHS.

Dense Granule Defect

A dense granule defect has been described in a family of American Cocker Spaniels.¹³ Affected dogs had a moderate to severe bleeding diathesis, particularly after trauma or surgery. Three of five affected dogs had impaired platelet aggregation responses to ADP and collagen. Platelet ¹⁴C-serotonin uptake and retention were normal; ATP content was also normal. ADP content was low resulting in an increased ATP/ADP ratio. Platelet morphology at the electron microscopic level was normal suggesting a functional dense granule defect. The disorder has not been recognized in Cocker Spaniels since the initial report. Molecular studies have not been conducted.

SIGNAL TRANSDUCTION DISORDERS

Calcium Diacylglycerol Guanine Nucleotide Exchange Factor I (CaDAG-GEFI) Thrombopathias

Basset hounds, Eskimo Spitz, Landseers of European Continental Type (ECT) and Simmental cattle have been identified with inherited signal-transduction platelet disorders that are virtually identical in presentation at

the clinical and functional level.^{4,15,26,27,29,45,49} Terms describing these disorders in the literature include canine thrombopathia, Basset hound thrombopathia, Spitz thrombopathia, Landseer thrombopathia, Bovine thrombopathia, and Simmental thrombopathia. Platelet aggregation responses to ADP and collagen are minimal to absent in affected animals. Platelet aggregation in response to thrombin is rate-impaired and is characterized by a lag phase, lengthening the time to complete aggregation to 4–6 minutes (normal dog platelets aggregate fully within 3 minutes). Because thrombopathic platelets do respond to thrombin, clot retraction assays are normal. Flow cytometric assays using antibodies to α IIb or β 3 show normal receptor density. CAP-1 binding is absent on ADP or PAF-activated thrombopathic canine platelets indicating that affected platelets either fail to bind fibrinogen or the α IIb- β 3 receptor fails to induce changes in the conformation of fibrinogen necessary for detection by this RIBS antibody.⁹ Platelet function disorders, likely due to problems with signal transduction, have been described in other breeds, including mixed-breed dogs.¹⁴ These disorders have not been evaluated at the molecular level. As observed in people,⁴³ signal-transduction disorders are probably the most common cause of inherited intrinsic platelet disorders in dogs and possibly other species. The molecular basis for the signal transduction-related platelet disorders in Basset hounds, Spitz, Landseers-ECT and Simmental cattle is due to distinct mutations in the gene encoding calcium diacylglycerol guanine nucleotide exchange factor I (CalDAG-GEFI).^{9,10} All of the mutations are within areas encoding structurally conserved regions (SCRs) of the catalytic domain (Tables 82.1, 82.2, and 82.3). CalDAG-GEFI is a signal transduction protein that functions as a guanine nucleotide exchange factor (GEF) and facilitates GDP/GTP switching by Rap1b. Rap-1b is a Ras-related low molecular mass guanine nucleotide binding protein (GTPase) that is activated by the binding of GTP. Activated Rap-1b is critically involved in activation and change of conformation of integrin α IIb- β 3 necessary for fibrinogen binding and ensuing platelet aggregation.³ The ability of thrombin to activate mutant platelets is thought to be attributable to the ability of PAR4 signaling to bypass CalDAG-GEFI and activate Rap1b directly or act via a pathway independent of Rap1b.^{9,20}

The mutation in Basset hounds is believed to be widespread; results of DNA testing at Auburn University indicate that between 25% and 30% of Basset hounds are carriers for the mutation. The mutation is

more prevalent in Basset hound lines used for show rather than for hunting. Landseer-ECT is a distinct breed recognized in Europe since 1960. Breeders within several European countries have tested their breeding stock for the CalDAG-GEFI mutation. The prevalence of the carrier state in Landseers-ECT, based on testing at Auburn University, is around 25%. Other than the original two Eskimo Spitz reported in 1994, no other affected or heterozygous Eskimo Spitz have been identified. Whether this small number results from a very low prevalence or lack of recognition of the disorder is not known. The prevalence of the CalDAG-GEFI gene mutation in Simmental cattle is also unknown; testing for the disorder has not been mandated by breed organizations. A mutation in CalDAG-GEFI was identified in an Arabian horse that presented with hemoabdomen and enlarged spleen at Auburn University (M.K. Boudreaux, personal observation). The mutation was a single nucleotide change in Exon 6 at coding nucleotide 687 (c.687C>G) and is predicted to result in the change of a histidine to a glutamine at position 229 (H229Q) in SCR2 (Table 82.3). Although this mutation was in an area encoding a highly conserved region and would be predicted to affect the structure of SCR2, platelet aggregation in response to ADP and collagen was only mildly diminished. The presence of this mutation may result in enhanced susceptibility of affected horses for abnormal hemorrhage, particularly if given platelet inhibitory medications or subjected to trauma.

To eliminate confusion in the literature and to more precisely describe the nature of the platelet disorders described in Basset hounds, Spitz, Landseers-ECT, and Simmental cattle they should be referred to as CalDAG-GEFI platelet disorders. Although CalDAG-GEFI is also expressed in brain, neurological signs in people or animals with CalDAG-GEFI disorders have not been reported. CalDAG-GEFI may play a role in signal transduction events in other hematopoietic cells, including neutrophils. Redundant pathways, however, are likely responsible for preventing significant impairment of neutrophil function in animals with CalDAG-GEFI mutations.

Kindlin-3

Recently a mutation in the gene encoding Kindlin-3 was identified in a German Shepherd dog referred to Washington State University (MK Boudreaux, personal observation). The dog had a history of prolonged bleeding, persistently high leukocyte counts, and susceptibility to infections. Kindlin-3 is a signal transduction

TABLE 82.3 Amino Acids within Structurally Conserved Regions (SCR) of the Catalytic Domain of CalDAG-GEFI

Species	SCR	Mutation ^a
Basset and Spitz	SCR1	<u>F</u> DHLEPLELAEHLTYEYRSECKI
Cow	SCR2	RAGVITHFVHVAEEL <u>L</u> LHLQNFNTLMAVVGGLSHSSISRLKETH
Horse	SCR2	RAAVITHFV <u>H</u> VAEKLLLELQNFNTLMAVVGGLSHSSISRLKETH
Landseer-ECT	SCR4	<u>R</u> LNGAK

^aUnderlined/bold amino acids are sites of mutations described in dogs, cattle, and horses.

protein shared by all hematopoietic cells that plays an essential role in integrin activation.

PROCOAGULANT EXPRESSION DISORDERS

Scott Syndrome

A family of German shepherd dogs has been described with a bleeding disorder resulting from lack of procoagulant expression on the surface of platelets, similar to Scott syndrome described in people.^{11,12} Affected dogs experienced intramuscular hemorrhage, epistaxis, and hyphema. Platelet morphology at the light microscopic level and platelet numbers were normal. Platelet aggregation and release, clot retraction, and buccal mucosa bleeding times were also normal. Affected platelets were markedly impaired in their ability to externalize membrane phosphatidylserine or generate platelet prothrombinase activity in response to collagen, thrombin, or combinations of collagen and thrombin. Platelet microvesicle generation was also impaired in response to calcium ionophore, thrombin, and collagen. In flow cytometric experiments, Annexin V binding by affected platelets activated by calcium ionophore was markedly reduced, supporting the inability of platelets to express phosphatidylserine. Although this is a platelet disorder, the clinical signs are more typical of a coagulopathy due to the inability of the platelets to generate and support a surface for coagulation protein activation. The molecular basis for this disorder is not known in dogs or in people.

DIAGNOSIS

The existence of inherited thrombocytopenia should be considered in dogs, particularly in CKCS, with persistent macrothrombocytopenia in the absence of clinical signs. Documentation of a mutation in the gene encoding β 1-tubulin is recommended to avoid subjecting these animals to unnecessary and potentially harmful drug treatments.

The existence of a qualitative intrinsic platelet disorder should be considered in animals with platelet-type bleeding (mucosal bleeding, epistaxis, petechial/ecchymotic hemorrhages) in the absence of thrombocytopenia (platelet numbers greater than 50,000/ μ L) and in the presence of normal coagulation screening assays and VWF factor antigen levels of greater than 15%. Diagnosis of novel intrinsic platelet disorders requires specialized testing which may include platelet aggregation and release, flow cytometry, and electron microscopy. Some of these techniques are not readily available and several require that the affected animal be on the premises of the testing facility for sample collection and processing. As a consequence, novel intrinsic platelet disorders are often diagnosed based on exclusion. In recent years the molecular basis for several of the documented inherited intrinsic platelet disorders in animals has been determined, not only facilitating diagnosis of

affected animals but also providing a means of identifying heterozygous individuals.

CASE MANAGEMENT

Clinical signs in animals with inherited intrinsic platelet disorders can vary considerably, even in animals with the same type of disorder. On a day to day basis hemorrhages are typically mild and sporadic, and tend to be self-limiting. Transfusions are usually only required in animals scheduled for surgery or subjected to major trauma. Minor procedures such as ear and teeth cleaning should be performed with extreme care. Hemorrhage into the ear canal can result in a vicious cycle of trauma and hemorrhage as a consequence of head shaking and scratching that can be difficult to curtail. Bleeding in the oral cavity may initially be missed if the animal swallows the blood; melena may be the first indication of this type of bleeding. Bloating can occur with excessive swallowing of blood and air. Gingival bleeding can be controlled using topical thrombin in animals with CalDAG-GEFI thrombopathies; however, repeated use of thrombin may result in immune reactions to thrombin and should be avoided. Surgical adhesive can also be used for any of the inherited platelet disorders to control bleeding from small skin lesions. Irritants present in cedar shavings, harsh detergents, cigarettes, or solvents, may elicit epistaxis and exposure should be avoided. Oral or topical medications that inhibit platelet reactivity or result in thrombocytopenia should also be avoided. Blood loss through the gastrointestinal or urinary tracts can be insidious and can result in iron deficiency anemia. Iron levels should be monitored in affected dogs less than a year old with chronic insidious bleeding. The animals should also be evaluated for intestinal parasites. It may be necessary to supplement iron using oral supplements and if oral iron is not sufficient to restore iron levels then injections should be considered using the smallest gauge needle to minimize risk of bleeding at injection sites. Affected dogs should be evaluated by a veterinarian at least every 6 months, even in the absence of apparent clinical signs. Owners can be taught to periodically assess mucous membranes for lightening in color as an indication of anemia.

Animals scheduled for surgery or subjected to trauma may require transfusion of multiple units of platelet-rich plasma or platelet concentrates. These products, which can be difficult and costly to obtain, cannot be stored for longer than 5 days and should be maintained at room temperature with gentle rocking to avoid platelet activation. Whole blood transfusions can be administered, but when given in volumes designed to prevent circulatory overload they may not provide enough functional platelets to produce effective hemostasis.

Repeated transfusions in animals with GT may result in an immune response against α IIb- β 3 on transfused platelets. In people with inherited platelet disorders alternative treatments have been evaluated including administration of 1-desamino-8-D-arginine vasopressin (DDAVP) and recombinant human factor VIIa.^{19,42} The

mechanism of action of DDAVP in shortening of bleeding times in people with inherited platelet disorders is thought to entail more than enhanced release of VWF from Weibel-Palade bodies; however, those mechanisms remain unclear. DDAVP was found to be more useful in people with platelet signal transduction and granule disorders than in people with GT; recombinant human factor VIIa was found to be more useful in the latter group. The use of non-plasma-derived reagents and species compatible purified plasma products in veterinary medicine is largely unexplored but may be attractive as an alternative method of treatment, particularly in dogs.

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Acquired Platelet Dysfunction

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Acronyms and Abbreviations

ADP, adenosine diphosphate; BMBT, buccal mucosal bleeding time; BVDV, bovine viral diarrhea virus; CCL5, chemokine L5; CKCS, Cavalier King Charles Spaniel; COX, cyclooxygenase; DIC, disseminated intravascular coagulopathy; EPO, erythropoietin; FDP, fibrin degradation products; FeLV, feline leukemia virus; IMT, immune-mediated thrombocytopenia; MR, mitral regurgitation; MVP, mitral valve prolapse; NSAIDs, non-steroidal anti-inflammatory agents; OHE, ovariohysterectomy; PAF, platelet activating factor; PFA-100, platelet function analyzer-100; PRP, platelet-rich plasma; TEG, thromboelastography; TXA₂, thromboxane A₂; VWD, von Willebrand disease; VWF, von Willebrand factor.

INTRODUCTION

Impaired platelet function should be considered in any patient with a bleeding tendency in the absence of thrombocytopenia, low von Willebrand factor (VWF) levels, or laboratory evidence of coagulopathy (e.g. prolonged prothrombin time, activated partial thromboplastin time, or activated clotting time).

Abnormal platelet function, also known as thrombopathia, thrombopathy, or thrombocytopathy, may be primary (congenital, usually inherited) or secondary (acquired). Inherited intrinsic platelet disorders and von Willebrand Disease (VWD) are discussed in other chapters in this text (see Chapters 81 and 82). Acquired disorders occur most commonly due to underlying disease or treatment with drugs or other exogenous agents. These conditions may be subcategorized into hypofunctional and hyperfunctional disorders, which may cause bleeding tendencies and thrombotic tendencies, respectively. Clinical manifestations of acquired

thrombopathies are highly variable. Ideally, therapy should be directed toward removing or managing the underlying disease process or exogenous stimulus causing secondary platelet dysfunction. Specific etiologies and mechanisms of acquired platelet dysfunction are discussed in more detail below.

ACQUIRED PLATELET HYPOFUNCTION

Acquired platelet hypofunction may occur secondary to underlying disease or treatment with drugs or other exogenous agents.

Underlying Disease

Many pathologic conditions are recognized as potential causes of impaired platelet function, including renal failure, anti-platelet antibodies, infections, malignancies, increased fibrinolytic products, and others.

Uremia

Uremia is recognized as a cause of platelet dysfunction in people and animals.^{29,34,61} Many molecules and pathways affecting platelet adhesion, aggregation, and secretion have been implicated.¹ An experimental model of renal failure in dogs resulted in prolonged buccal mucosal bleeding time and markedly impaired platelet glass bead retention, but did not significantly affect platelet concentration, volume, or aggregation in response to several agonists, or coagulation assays (prothrombin time and activated partial thromboplastin time), findings consistent with defective platelet adhesion.¹⁵ Neither quantitative nor qualitative abnormalities in VWF have been shown consistently in people with uremia, suggesting that the impaired adhesion in the uremic dogs was due to abnormal platelet function.¹

Anti-platelet Antibodies

Anti-platelet antibodies not only may cause immune-mediated thrombocytopenia (IMT), but are also recognized as a cause of platelet dysfunction. Specifically, antibodies against GPIIb-IIIa ($\alpha_{IIb}\beta_3$) and other key platelet surface receptors such as Ib-IX-V have been implicated in people.¹ Experimental work in dogs and equids suggests a similar phenomenon. A study in which serum from normal dogs or dogs with IMT was added to platelet-rich plasma (PRP) from a normal dog found the serum from dogs with IMT to cause impaired aggregation to several platelet agonists.⁵² In another study, serum from dogs experimentally infected with *Ehrlichia canis* inhibited aggregation of platelets from a normal dog.⁴³ Serum from mule foals with experimentally-induced neonatal alloimmune thrombocytopenia had evidence of higher concentration of platelet-associated IgG than serum from control mule foals, and addition of serum from affected foals to donkey PRP caused impaired aggregation in response to collagen.⁷⁷ Exogenous anti-platelet antibodies also comprise a group of drugs designed to inhibit platelet function (see "Platelet Inhibitors", below).

Infection, Inflammation, and Neoplasia

There is evidence that type II bovine viral diarrhea virus (BVDV), in addition to causing thrombocytopenia, may also impair platelet function. Experimental work in neonatal calves showed impaired platelet aggregation in affected animals.^{93,95} In contrast, work by the same research group found no evidence of altered platelet function in cattle persistently infected with BVDV.⁹⁴

Feline leukemia virus (FeLV) can infect megakaryocytes and, in addition to causing many other hematologic abnormalities, may cause platelet abnormalities potentially associated with impaired function.⁸¹ Clear evidence of a causal relationship between FeLV and platelet hypofunction is lacking. However, abnormal platelet or megakaryocyte morphology is a feature of

many FeLV-related conditions, both neoplastic and non-neoplastic, and it is plausible that this has a functional correlate.^{6,13}

There is conflicting information about the effects of endotoxin or sepsis on platelet function, with data to suggest both decreased and heightened reactivity.⁹² Lipoteichoic acid from *Staphylococcus aureus* has been shown to impair intracellular calcium mobilization and aggregation in human platelets.⁸² *Yersinia pestis* expresses a surface protein, YopM, that is structurally similar to the VWF- and thrombin-binding domain of the platelet GPIb receptor, and has been shown to inhibit aggregation of human platelets, possibly via competitive binding with thrombin.⁵⁴ The clinical significance of these findings is not clear.

Studies of experimentally-induced pancreatitis in dogs have found evidence of both platelet hypofunction and hyperfunction. In one study, pancreatitis caused impaired platelet aggregation in response to several agonists and reduced ATP release in response to collagen.⁴⁶ Another study found platelets to have increased sensitivity to ADP and PAF within the first hour after the insult to the pancreas, but diminished or normal sensitivity thereafter.⁵⁶

Hyperglobulinemia, either due to antigenic stimulation (e.g. ehrlichial) or neoplasia (e.g. multiple myeloma), is commonly recognized in veterinary medicine as a potential cause of impaired platelet function, possibly by "coating" the platelet surface and impairing adhesion and aggregation.^{29,87,91} Platelet dysfunction is a recognized complication of IgA and IgG myeloma and Waldenström macroglobulinemia in people, and some in vitro experiments have shown paraprotein to be capable of interfering with platelet function.¹ A dog with IgA myeloma had a mildly prolonged bleeding time but more specific platelet function assays were not performed.⁷⁹

Increased Fibrinolytic Products and Liver Disease

It is not clear how often or to what degree increased plasma concentration of fibrin degradation products (FDPs) causes platelet inhibition in the clinical setting. Purified low molecular mass fibrinogen degradation products have been shown to inhibit platelet aggregation in vitro, but may not occur in high enough concentrations in vivo to produce this effect, and patients with disseminated intravascular coagulopathy (DIC) or other conditions may have decreased in vitro platelet aggregation due to in vivo platelet activation.^{74,86} Nevertheless, increased plasma concentration of FDPs is commonly cited as a potential cause of impaired platelet function in animals.^{29,87,91} Increased FDPs may occur due to increased fibrinolysis (e.g. in DIC) or decreased clearance (with liver insufficiency). Certainly, factors other than FDPs contribute to a bleeding tendency in patients with DIC. A study of dogs with liver disease found impaired platelet aggregation in response to some agonists, and bleeding tendencies consistent with thrombopathy, but FDPs were not shown to be the causative agent, and experimentally-induced hyperammonemia

has been shown to inhibit platelet aggregation in rats.^{83,106}

Snake Envenomation

Snake venoms contain complex mixtures of bioactive molecules (e.g. proteases, phospholipases, disintegrins, lectins) that can affect hemostasis in many ways, including interference with platelet function via interaction with receptors, modulation of ADP release or TXA₂ formation, and production of reactive oxygen species.^{23,69} Experimental work in animals has shown species-dependent platelet sensitivity to some snake venom proteins.⁵⁵

Drugs and Other Exogenous Agents

Many drugs and other exogenous agents have been shown to have inhibitory effects on platelet function. Some of these agents have platelet inhibition as their main clinical application, while others cause platelet inhibition as a side-effect.

Platelet Inhibitors

Some drugs are designed to inhibit platelet function. Most of these are platelet receptor antagonists. Examples that have been used in animals include peptides or antibodies that inhibit GPIIb/IIIa ($\alpha_{IIb}\beta_3$ integrin) and agents that inhibit the ADP receptor.^{4,5,9,16,17,24,25,27,33,45,98,100,103,109} New platelet inhibitors continue to be developed for use in people.^{2,3}

Anti-inflammatory Agents

Many anti-inflammatory agents inhibit platelet function. The main inhibitory mechanism is inactivation of the cyclooxygenase (COX) enzyme, which results in decreased production of the potent platelet agonist, thromboxane A₂ (TXA₂). Aspirin, which irreversibly inactivates both COX-1 (expressed by both newly formed and mature platelets) and COX-2 (expressed only by immature platelets) isoenzymes, has been shown to inhibit platelet function in many animal models.^{8,14,48,63,64,75} Platelet responses to aspirin evidently are species-dependent. In some species, aspirin impairs platelet aggregation in response to collagen and ADP. In cattle, however, treatment with oral aspirin resulted in decreased TXA₂ production but did not impair platelet aggregation in response to collagen, ADP, or PAF.³⁷ Aspirin also does not inhibit ADP-induced platelet aggregation in most dogs. This is likely because platelet release is not a necessary co-component of ADP-induced irreversible platelet aggregation in most dogs.^{7,8,10} Aspirin will inhibit ADP-induced aggregation responses in the small subset of dogs in which ADP-induced platelet aggregation is accompanied by dense granule release.

Other non-steroidal anti-inflammatory agents (NSAIDs) inactivate COX reversibly, and some of them

selectively inhibit COX-2. Phenylbutazone has been shown to inhibit platelet aggregation in horses for a short time after administration, without causing a prolongation in BMBT.⁶² A recent study evaluating effects of several non-aspirin NSAIDs in dogs found that carprofen inhibited platelet aggregation, and caused thromboelastography (TEG) changes suggesting hypocoagulability, but deracoxib and meloxicam did not; in fact, deracoxib caused TEG changes consistent with increased clot strength.¹⁴ However, information on effects of carprofen on platelet function in dogs is conflicting; other studies found no evidence of carprofen causing impaired aggregation or prolonged PFA-100 closure time.^{35,42} Ketoprofen has been found to impair platelet aggregation and prolong PFA-100 closure time in dogs.^{35,42} The clinical implications of this effect are likely to be mild. In another study, dogs given ketoprofen before ovariohysterectomy (OHE) had inhibited platelet aggregation but did not have prolonged BMBT; the authors concluded that ketoprofen was safe to give to healthy dogs pre-OHE, but any such dogs should be screened preoperatively for bleeding problems and closely monitored after surgery.⁵³

Other Drugs and Exogenous Agents

There is a long list of other drugs or exogenous agents found to inhibit platelet function in animals or people. A partial list includes antibiotics (penicillins and cephalosporins); antihistamines; barbiturates; calcium channel blockers; chondroprotective agents; dextran 70; diethyl-carbamazine; dipyridamole; fibrinolytic and anti-fibrinolytic agents; halothane; heparin; hydroxyethyl starch; local anesthetics; marine fatty acids; mycotoxins; propamolol; psychotropic drugs; radiographic contrast agents; and wine or grape juice.^{1,8,19,20,26,28,30,36,39,48,49,51,58,60,65,70,72,105}

ACQUIRED PLATELET HYPERFUNCTION

Underlying disease or drug treatment may result in secondary platelet hyperfunction as well as hypofunction. Some studies in animals have found an association between underlying pathology or strenuous exercise and platelet activation, without demonstrating that the platelet response to the stimulus is abnormal.^{67,68,80,97,99,102} This section will be limited to conditions shown to result in abnormally enhanced platelet reactivity.

Underlying Disease

Many pathologic conditions are recognized as potential causes of enhanced platelet reactivity, including infectious and inflammatory diseases, neoplasia, and others.

Infection, Inflammation, and Neoplasia

Several infectious diseases are associated with increased platelet reactivity in animals, including heartworm disease and Rocky Mountain spotted fever in dogs,

feline infectious peritonitis in cats, and pasteurellosis in cattle.^{7-12,22,41,71} Platelets from experimentally infected animals have been found to be hyperaggregable in response to some agonists, although the mechanisms responsible for this enhanced reactivity are not well understood. There is conflicting data in the scientific literature about the effects of sepsis on platelet function in people, with evidence to suggest both inhibition and activation.⁹²

Interleukin-6, in addition to stimulating thrombopoiesis, increases the sensitivity of canine platelets to activation by some agonists.^{18,76} Ponies with experimentally-induced laminitis from excess dietary carbohydrate had increased platelet aggregation and formation of platelet-neutrophil aggregates.^{100,101} However, there is evidence indicating that inflammation may impair as well as enhance platelet reactivity. As noted above, studies of dogs with experimentally-induced pancreatitis have found evidence of both platelet hypofunction and hyperfunction. Moreover, a study using bovine samples found that addition of C-reactive protein to platelets suspended in homologous plasma impaired aggregation in response to some agonists.²¹

Cancer may be a cause of acquired platelet hyperfunction. Platelet hyperaggregability was detected in two dogs with probable essential thrombocythemia.³¹ A study in dogs found that platelets from dogs with cancer (not all of which were hematologic malignancies) were more sensitive to some agonists, compared to platelets from control animals.⁵⁹

Taurine Deficiency in Cats

The effect of taurine deficiency on platelet function in cats has been the subject of investigation, at least in part because of the tendency for cats with cardiomyopathy associated with taurine deficiency to develop thromboembolic disease. One aggregometry study using collagen as an agonist found platelets from taurine-depleted cats to be hyperaggregable compared to platelets from cats receiving taurine, and platelets from people supplemented with taurine to be hypoaggregable compared to platelets from people with normal taurine status.⁴⁴ However, another similar study in cats did not confirm these findings, showing mildly increased collagen-induced serotonin release but no difference in collagen-induced platelet aggregation between taurine-deficient and taurine-replete cats; moreover, in this study there was evidence of decreased ADP-induced platelet aggregation and serotonin release.¹⁰⁴ A recent study using the PFA-100 instrument showed no evidence of shorter closure times in cats with hypertrophic cardiomyopathy, compared to normal cats.⁴⁷

Heart Disease

Mitral valve disease is associated with increased reactivity and decreased survival of platelets in humans, and a possible association between mitral valve disease and platelet dysfunction has been the subject of inves-

tigation in dogs, particularly in Cavalier King Charles Spaniels (CKCS).⁷³ Congenital platelet abnormalities in CKCS, as discussed in more detail in Chapter 82, complicate interpretation of platelet function testing results in this breed. One study in CKCS found no correlation between degree of mitral valve prolapse (MVP) and platelet aggregation responses.⁷³ Another study found CKCS with MVP had enhanced aggregation responses regardless of mitral regurgitation (MR) status, but platelets did not appear to circulate in an activated state. In that study, CKCS with MR and dogs with subaortic stenosis had prolonged PFA-100 closure times but also had lower proportions of large VWF multimers, likely secondary to consumption under high shear conditions, suggesting a mechanism of impaired primary hemostasis other than platelet hypofunction.⁹⁰

Nephrotic Syndrome

Enhanced platelet responsiveness to some agonists has been described in people and dogs with nephrotic syndrome, and may increase the risk of thromboembolic complications in these patients.^{40,57,78,84} The basis of the enhanced sensitivity is probably multifactorial; one study found that hyperaggregability can be inhibited *in vitro* by increasing plasma albumin concentration.⁴⁰

Asthma

A study of people with asthma found increased sensitivity of platelets to some agonists, as evidenced by increased intracellular free calcium concentration, increased CD62P expression, and increased plasma concentration of the RANTES or CCL5 cytokine.⁶⁶ This enhanced response was inhibited in people with asthma who were taking oral theophylline. It is not clear whether allergic respiratory disease in animals causes similar platelet hyperfunction.

Diabetes Mellitus

Diabetes is a risk factor for atherosclerosis in people, likely due in part to altered platelet function.^{32,89,96} Platelet hyperreactivity in people with diabetes probably occurs due to multiple mechanisms, including glycation of proteins, oxidative damage to lipids, increased GP Ib-IX receptor expression, and altered signal transduction pathways.^{32,85} Effects of diabetes on platelet function are evident in the laboratory by enhanced sensitivity to agonists. In one study, platelets from people with diabetes had increased adhesion to an extracellular matrix substrate composed of collagen, adhesive glycoproteins, and proteoglycans, but not VWF.⁵⁰ Rats with streptozotocin-induced diabetes had markedly increased release of arachidonic acid in response to thrombin in one study, and enhanced aggregation to ADP and thrombin and increased release of serotonin in response to thrombin in another study.^{38,107} The prevalence and clinical significance of altered platelet function in domestic animals with diabetes is not known.

Drugs and Other Exogenous Agents

In dogs, administration of erythropoietin (EPO) increased platelet responsiveness to thrombin and increased the proportion of reticulated platelets.¹⁰⁸ A study in humans found that EPO increased platelet reactivity but did not change the numbers of reticulated platelets.^{88,108} Platelet reactivity was increased after thiacetarsamide treatment of Beagles experimentally implanted with adult heartworms, but not after treatment of dogs with naturally occurring heartworm infections.⁷

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A microscopic image of a blood smear, showing various types of white blood cells (leukocytes) and platelets. The cells are stained, likely with Wright's stain, showing different colors and shapes. The background is a light blue color.

SECTION VII

Hemostasis

Marjory B. Brooks

Overview of Hemostasis

STEPHANIE A. SMITH

Enzyme Biochemistry

- The Nature of Enzymatic Cascades
- Enzymatic Complexes (Cofactors and Enzymes)
- Inhibition of Enzymatic Activity
- Membrane Surface Interactions

Coagulation Proteins that Generate Thrombin

Contact Pathway (Kallikrein-Kinin Pathway)

- Factor XII
- Prekallikrein
- Kininogens

Extrinsic (Tissue factor) Pathway

- Tissue factor
- Factor VII

Intrinsic Pathway

- Factor XI
- Factor IX
- Factor VIII

Common Pathway

- Factor X
- Factor V
- Prothrombin

Coagulation Inhibitors

- Tissue factor pathway inhibitor
- C1-Inhibitor
- Antithrombin
- Protein C pathway
- Miscellaneous inhibitors

Cells that Participate in Hemostasis

- Endothelial Cells
 - Antithrombotic properties of endothelium

- Prothrombotic properties of endothelium
- Other properties of endothelial cells

Platelets

Tissue Factor Bearing Cells

Microparticles

The Role of Cell Surface Membranes

Cell Based Model of Thrombin Generation

Initiation

Amplification

Propagation

Thrombin

Feedback Regulation of Coagulation

Platelet Activation

Fibrin Formation

Activation of Factor XIII

Fibrinogen and Fibrin Clot Structure

Fibrinolysis

Plasminogen

Plasminogen Activators

Tissue-type plasminogen activator

Urokinase plasminogen activator

Fibrinolysis Inhibitors

Plasminogen activator inhibitor 1

α_2 -Antiplasmin

Thrombin activatable fibrinolysis inhibitor

Miscellaneous inhibitors

Hemostasis and Inflammation

Effects of Hemostasis on Inflammation

Effects of Inflammation on Hemostasis

Acronyms and Abbreviations

α_1 -PI, α_1 -Protease inhibitor; α_2 -MG, α_2 -macroglobulin; α_2 -AP, α_2 -antiplasmin; APC, activated protein C; AT, anti-thrombin; C1-INH, C1-inhibitor; C4BP, C4 binding protein; DDAVP, desamino-8-D-arginine vasopressin; EPCR, endothelial protein C receptor; ER, endothelial reticulum; FV/FVa, factor V/activated factor V; FVII/FVIIa, factor VII/activated factor VII; FVIII/FVIIIa, factor VIII/activated factor VIII; FIX/FIXa, factor IX/activated factor IX; FX/FXa, factor X/activated factor X; FXI/FXIa, factor XI/activated factor XI; FXII/FXIIa, factor XII/activated factor XII; FXIII/FXIIIa, factor XIII/activated factor XIII; FDP, fibrin degradation products; FpA, fibrinopeptide A; FpB, fibrinopeptide B; Gla, gammacarboxyglutamic acid; GPI, glycosylphosphatidylinositol; HC, heparin cofactor II; HK, high molecular weight kininogen; HSPG, heparan sulfate proteoglycan; LRP, low density lipoprotein receptor-related protein; MP, microparticle; NO, nitric oxide; PAF, platelet activating factor; PAI-1, plasminogen activator inhibitor 1; PARs, protease activated receptors; PC, protein C; PChol, phosphatidylcholine; PCI, protein C inhibitor; PEth, phosphatidylethanolamine; PGI₂, prostacyclin; PK, prekallikrein; PLG, plasminogen; PS, protein S; PSer, phosphatidylserine; PZ, protein Z; sctPA, single chain tissue plasminogen activator; scuPA, single chain urokinase

plasminogen activator; serpin, serine protease inhibitor; TAFI/TAFIa, thrombin activatable fibrinolysis inhibitor/activated thrombin activatable fibrinolysis inhibitor; TF, tissue factor; TFPI, tissue factor pathway inhibitor; TM, thrombomodulin; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor; VWF, von Willebrand factor; ZPI, Protein Z-dependent protease inhibitor.

Hemostasis is a vital protective mechanism that prevents blood loss by sealing sites of injury in the vascular system. Hemostasis must be controlled, however, so that blood does not coagulate within the vasculature. Such uncontrolled coagulation restricts normal blood flow, resulting in tissue hypoxia.

Hemostasis consists of a tightly controlled and well-balanced interplay among a large number of cellular and protein participants. Endothelial cells lining the vasculature, cells outside the vasculature, and platelets all play important roles. Hemostatic proteins include inactive precursors (zymogens) that are converted to active enzymes, regulatory proteins (cofactors) that enhance the functionality of their corresponding coenzymes, and inhibitors that interfere with protein function through a variety of mechanisms (see Table 84.1).

Normally, coagulation does not occur within the vasculature because properties of the resting cellular participants render them inactive, and because the majority of protein participants circulate in their inert, zymogen form. The initiation of coagulation in response to injury depends on the exposure of extravascular components that are not present within the bloodstream under physiologic conditions. Exposure of these extravascular participants initiates an explosive cascade of cellular activation, changes in cell-surface properties, and generation of active enzymes that produce a stable clot. Some of the cells and enzymes also trigger vascular wall responses, inflammatory pathways, and immune-defense mechanisms. Coagulation also initiates fibrinolysis, the process by which the clot is removed to restore blood flow. Ultimately, coagulation triggers the cell migration and proliferation that promote healing.

ENZYME BIOCHEMISTRY

The Nature of Enzymatic Cascades

The clotting cascade consists of a series of enzymatic reactions. Protein participants circulate in the blood mostly as inert precursors, or zymogens. These zymogens are activated by limited proteolysis to form active enzymes. With the exception of thrombin, all of these enzymes have limited activity unless they bind to their protein cofactor and a procoagulant membrane surface. Once the complete enzymatic complex has assembled on a suitable membrane surface, the activity of the enzyme is markedly exaggerated, by as much as several hundred-thousand fold.

Each cascade reaction is initiated when the active enzyme complex binds to its substrate, resulting in cleavage of the substrate to generate the next enzyme in the series. In many cases, positive feedback occurs

because a downstream enzyme is able to back-activate an upstream zymogen, thereby markedly amplifying the generation of downstream enzymes from minimal amounts of upstream enzymes. Negative feedback is also a common feature in the coagulation cascade, with downstream enzymes generating inhibitors that down-regulate activity of upstream enzymes. The generation of a blood clot is carefully controlled through the complex interplay of the initial trigger, amplification, and inhibition.

Enzymatic Complexes (Cofactors and Enzymes)

The majority of enzymes participating in coagulation are serine proteases with homology to trypsin, the prototype enzyme of this class. Serine proteases cleave after arginine residues. Other coagulation proteins have no enzymatic capabilities, but rather play regulatory roles in enhancing enzymatic activity. These proteins circulate in inactive forms, called procofactors. A procofactor must be cleaved by the appropriate enzyme to result in a fully active cofactor. Proteolytic activation of zymogens to enzymes and procofactors to cofactors can take several forms (Fig. 84.1).

Many of these proteins contain disulfide bonds between two cysteine residues within the protein. Enzymatic cleavage of an activation site that lies between the cysteine residues will convert the protein from a single chain protein to a two chain protein of identical molecular weight, without removing any of the amino acid sequence.

When the activation site does not lie between disulfide bound cysteine residues, cleavage of a single site will result in two different fragments of the initial protein. The smaller non-functional fragment is the activation peptide, while the remaining fragment is the fully activated enzyme or cofactor.

Some of the proteins contain multiple activation sites. If both lie between disulfide-bridged cysteine residues, then an activation peptide is released and a two-chain enzyme is produced. The order in which the target sites are cleaved may result in inactive intermediates, or partially or fully active intermediate enzymes. Complete cleavage of all of the sites will generally result in multiple fragments.

Fully functional enzymatic complexes often consist of both an enzymatic subunit (the serine protease) and a regulatory subunit (the cofactor).

Inhibition of Enzymatic Activity

Coagulation and fibrinolysis enzymes may be inhibited through several general mechanisms: cleavage of the

TABLE 84.1 Hemostatic Proteins, Factors, Cofactors, and Inhibitors

Functional Classification	Abbreviation	Common Use Name	Previous Names	Characterization
Extrinsic pathway	TF	Tissue Factor	Thromboplastin	Cofactor
	FVII	Factor VII	Proconvertin	Zymogen
	FVIIa	Factor VIIa		Enzyme
Contact pathway	FXII	Factor XII	Hageman factor	Zymogen
	FXIIa	Factor XIIa		Enzyme
	PK	Prekallikrein	Fletcher factor	Zymogen
	K	Kallikrein		Enzyme
	HK	High molecular weight kininogen	Williams-Fitzgerald-Flaujeac factor	Cofactor
Intrinsic pathway	FXI	Factor XI	Plasma thromboplastin antecedent	Zymogen
	FXIa	Factor XIa		Enzyme
	FIX	Factor IX	Christmas factor	Zymogen
	FIXa	Factor IXa		Enzyme
	FVIII	Factor VIII	Antihemophilic factor	Procofactor
FVIIIa	Factor VIIa			Cofactor
Common	FX	Factor X	Stuart-Prower factor	Zymogen
	FXa	Factor Xa		Enzyme
	FV	Factor V	Labile factor	Procofactor
	FVa	Factor Va		Cofactor
	Pro	Prothrombin	Factor II	Zymogen
	Fg	Fibrinogen	Factor I	Substrate
	Fb	Fibrin		Final product
	FXIII	Factor XIII		Zymogen
	FXIIIa	Factor XIIIa		Enzyme
Coagulation inhibitors	α_1 PI	α_1 -Protease inhibitor	α_1 -Antitrypsin	
	AT	Antithrombin	Antithrombin III	Serpin
	HC	Heparin Cofactor II		Serpin
	TFPI	Tissue factor pathway inhibitor	Extrinsic pathway inhibitor	Kunitz inhibitor
	PC	Protein C		Zymogen
	APC	Activated protein C		Enzyme, inhibitor
	PS	Protein S		Cofactor
	TM	Thrombomodulin		Cofactor
	PZ	Protein Z		Cofactor
	ZPI	Protein Z dependent protease inhibitor		Enzyme
Fibrinolysis	PLG	Plasminogen		Zymogen
	PLM	Plasmin		Enzyme
	scUPA	Single chain urokinase plasminogen activator	Prourokinase	Zymogen (slight enzyme)
	tcUPA (uPA)	Two-chain urokinase plasminogen activator	Urokinase	Enzyme
	sctPA	Single chain tissue-type plasminogen activator		Enzyme
	tctPA (tPA)	Two-chain tissue-type plasminogen activator		Enzyme
Fibrinolysis inhibitors	α_2 -AP	α_2 -Antiplasmin	Plasmin inhibitor	Serpin
	PAI-1	Plasminogen activator inhibitor-1		Serpin
	TAFIa	Thrombin activatable fibrinolysis inhibitor	Carboxypeptidase U, B, or R	Enzyme
Nonspecific inhibitors	C1-INH	C1 inhibitor		Inhibitor
	α_2 -MG	α_2 -Macroglobulin		Inhibitor
	PN1	Protease nexin 1		Inhibitor
	PCI	Protein C inhibitor	Plasminogen activator inhibitor 3	Serpin

enzyme, blockage of the active site, formation of stable complexes, and modification of the substrate (Fig. 84.2).³⁹

Some inhibitors are enzymes that act by cleaving the target protein, cutting it into pieces that are no longer

functional. An example of this kind of inhibition is the cleavage of activated factor V (FVa) by activated protein C (APC).

Blockage of the active site is an inhibitory mechanism that is commonly employed by the pharmaceutical

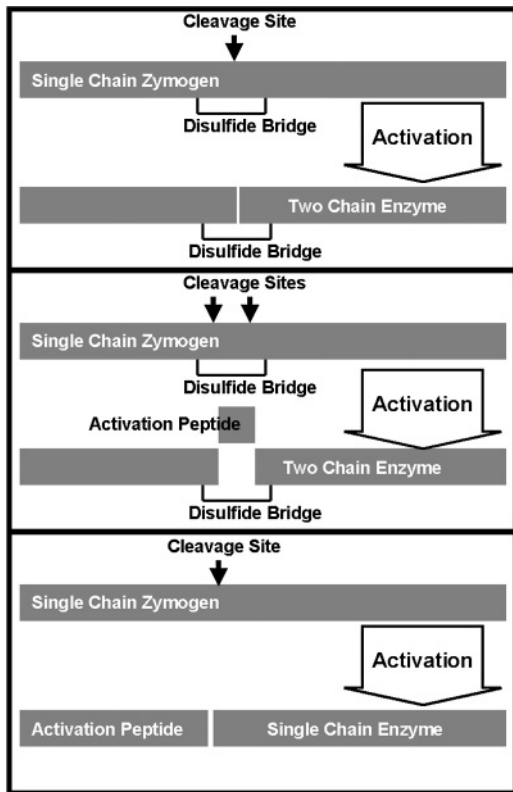


FIGURE 84.1 Enzymatic activation of coagulation proteins. Most coagulation proteins circulate as inactive, single chain polypeptides containing disulfide bridges between nonadjacent cysteine residues. Depending on the location of the disulfide bridges and the cleavage site (or sites), enzymatic cleavage may result in a two-chain activated protein of identical size to the precursor, a two-chain activated protein (smaller than the precursor) and a released activation peptide, or a single chain activated protein (smaller than the precursor) and a released activation peptide.

industry and by some naturally occurring inhibitors. Small molecule inhibitors may fit directly into the enzymatic site, limiting the access of the substrate.

Other inhibitors act by binding to the enzyme and altering it so it can't be released. As a result, the target enzyme and inhibitor form a stable complex that permanently inactivates the enzyme. An example of this mechanism is inhibition of thrombin by anti-thrombin-heparin. Antithrombin (AT) is a member of the serpin (serine protease inhibitor) class of inhibitors. These inhibitors employ a "suicide-substrate" mechanism. They contain a small peptide loop that acts as "bait" that mimics substrates of the target enzyme. However, binding of the target enzyme to the bait results in serpin structural modifications that shift the position and conformation of the enzyme.

A few inhibitors work by changing the nature of the substrate, so that the enzyme cannot bind effectively to its target site. The thrombin activatable fibrinolysis inhibitor (TAFI) acts via this mechanism by cleaving fibrin to limit plasmin-mediated fibrinolysis.

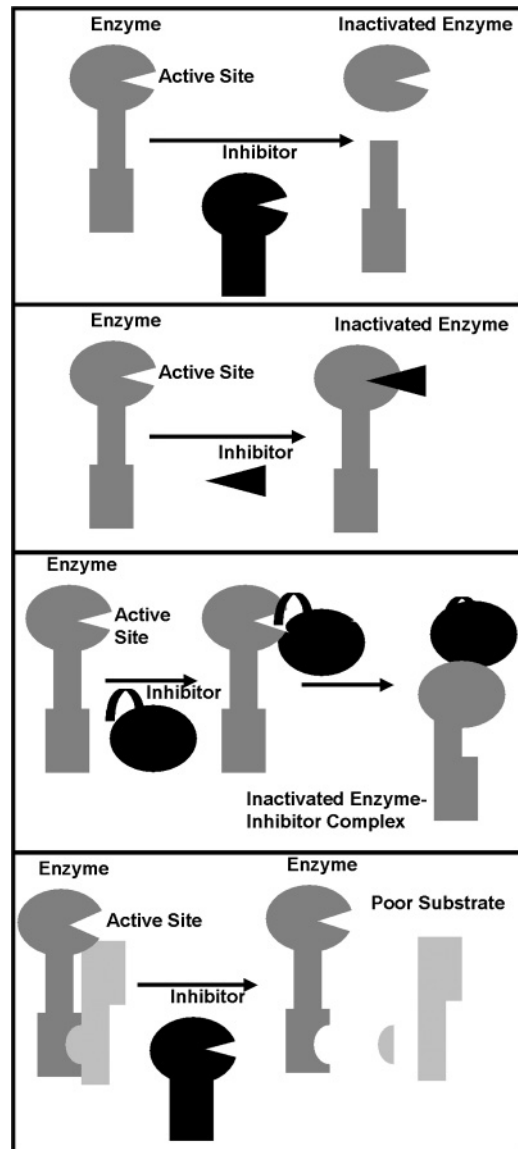


FIGURE 84.2 Coagulation enzyme and cofactor inhibition.

Inhibitors work through a variety of different mechanisms:

(1) Activated coagulation proteins can be inhibited by enzymes that cleave the target protein, damaging the structure and resulting in an inactivated protein. (2) Active site inhibitors bind the enzymatic site of the target enzyme, preventing the protease from cleaving its target protein. (3) Other inhibitors, such as serpins, display a "bait" peptide that looks like a substrate for the target enzyme. When the enzyme binds to and attempts to cleave the bait, the inhibitor changes in structure, resulting in a shift in position and structure of the target enzyme, which inactivates the enzyme into a stable complex with the inhibitor. (4) Some inhibitors modify the substrate rather than the target enzyme. These changes usually decrease binding interactions between the enzyme and the substrate, slowing or eliminating the ability of the enzyme to cleave its substrate.

Membrane Surface Interactions

The rate of coagulation is profoundly affected by the presence of appropriate membrane surfaces for enzymatic reactions. Membrane-binding enhances enzyme

activity because localization to a membrane surface helps properly align the participating proteins. Tissue factor (TF) is the only coagulation protein that is permanently attached to the membrane surface.³⁶

Some coagulation proteins (factors VII, IX, X [FVII, FIX, FX]), prothrombin, proteins C, S, Z [PC, PS, PZ]) contain gammacarboxyglutamic acid (Gla) residues that enable protein binding to a membrane surface via interaction with calcium and negatively charged phospholipids. Calcium-binding of the Gla domains of these proteins requires that the glutamic acid residues are carboxylated via intra-hepatic post-translational modifications involving the vitamin K cycle (Fig. 84.3).

Incompletely carboxylated Gla proteins do not properly bind calcium and are unable to assemble on an activated membrane surface. The importance of carboxylation in membrane binding is dramatically illustrated by the profound adverse impact of vitamin K antagonists such as warfarin (See Chapter 85). The

requirement of calcium for membrane-binding explains the ability of calcium chelators (e.g. citrate and EDTA) to prevent activation of many coagulation enzymes.

Some cofactors (factors V and VIII [FV and FVIII]) also have regions that interact with phospholipids, allowing fully functional enzymatic complexes to assemble on a membrane surface. The mechanism of membrane binding is less well described than that for Gla proteins, but involves the stereochemical configuration of the phosphatidylserine (Pser) head group.^{21,23}

COAGULATION PROTEINS THAT GENERATE THROMBIN

Previous coagulation models divided coagulation into the extrinsic, intrinsic and common pathways (Table 84.1, Fig. 84.4). The extrinsic pathway consisted of generation of active factor X (FXa) by the complex of TF

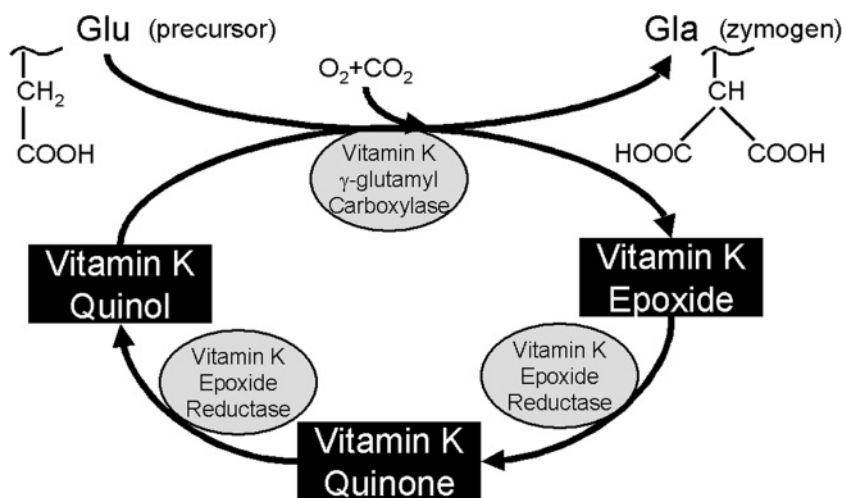


FIGURE 84.3 The vitamin K cycle. In hepatocytes this cycle is necessary for production of fully functional vitamin K dependent coagulation proteins. These proteins (prothrombin, FVII, FIX, FX, protein C, protein S, protein Z) are produced as precursors containing glutamic acid residues. The enzyme vitamin K γ -glutamyl carboxylase modifies these residues to γ -carboxyglutamic acid (Gla), producing zymogens containing Gla residues which are capable of binding Ca^{2+} and mediating membrane interactions. As a result of this enzymatic action, the vitamin K is converted to an epoxide, and must be regenerated via the activity of vitamin K epoxide reductase. Warfarin (and its derivatives) interfere with vitamin K epoxide reductase, preventing recycling of vitamin K epoxide to vitamin K quinol.

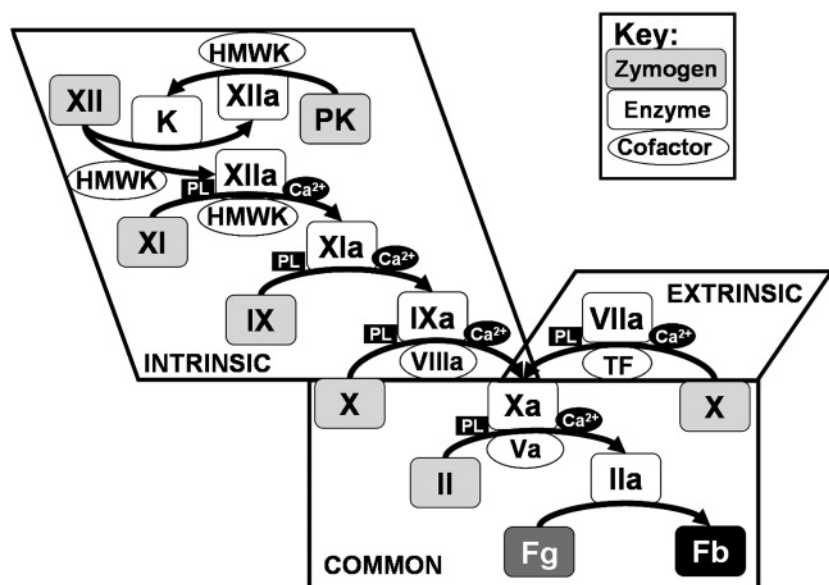


FIGURE 84.4 The cascade model of fibrin formation. This outdated model divides the coagulation system into separate, seemingly redundant pathways (extrinsic and intrinsic) either of which can result in the generation of FXa. The common pathway consists of formation of the prothrombinase complex (FXa-FVa) and results in generation of thrombin, followed by cleavage of fibrinogen to form fibrin. Many of the enzymes and enzymatic complexes require calcium (Ca^{2+}) and binding to active membrane surfaces (PL) for full activity. For simplicity, inhibitors of the various enzymes and feedback activation of procofactors to cofactors have been omitted. See Table 84.1 for abbreviations.

and activated factor VII (TF-FVIIa). The terminology “extrinsic” pathway arose from the requirement for TF (an extravascular, cell-bound protein) as a clotting trigger. The intrinsic pathway consisted of contact activation of factor XII (FXII), followed by a cascade consisting of activation of the downstream proteases factor XI (FXI) then FIXa-FVIIIa, ultimately resulting in FXa generation. This pathway was termed the “intrinsic” pathway due to the fact that it appeared to be intrinsic to blood, not requiring the addition of any extravascular trigger. The downstream portion of this cascade is still referred to as the intrinsic pathway, even though it is not truly intrinsic to blood. The common pathway consisted of the formation of the FXa-FVa complex, which generates thrombin. Thrombin then cleaves fibrinogen to fibrin, which spontaneously polymerizes into an insoluble fibrin gel.⁹ These classifications have been useful for *in vitro* laboratory evaluation, but such divisions do not occur *in vivo*.

Contact Pathway (Kallikrein-Kinin Pathway)

The contact pathway consists of the zymogens FXII (Hageman factor) and prekallikrein (PK), and the cofactor high molecular weight kininogen (HK; see Table 84.1). Activation of the two zymogens occurs spontaneously (auto-activation) upon assembly of the proteins on a suitable negatively charged surface.⁷ Because FXIIa can cleave downstream coagulation proteases, contact activation ultimately results in generation of thrombin and therefore formation of a fibrin clot. Consequently, contact activation (i.e. creation of the enzymes FXIIa and kallikrein) occurs whenever blood comes in contact with an artificial surface. Contact activation always occurs, to some extent, when blood is removed from the vascular system. The contact pathway is the source of blood’s apparent “intrinsic” clotting potential, and the reason blood coagulates when collected into a syringe or tube in the absence of anticoagulants. The amount of FXIIa and kallikrein generated depends on the negative charge of the artificial surface. Therefore, blood clots relatively slowly in plastic, more quickly in glass, and most rapidly

when in contact with a strong negative charge. Note that contact activation is *not* calcium dependent, and consequently is not abolished by collection of blood into calcium chelators such as citrate or EDTA.

Traditional coagulation cascade models often depict the contact pathway and the extrinsic pathway as independent and redundant generators of FXa, and subsequent thrombin and fibrin production (see Fig. 84.4). Recent advances clearly indicate that the contact pathway, while an important feature of *in vitro* coagulation, is irrelevant for *in vivo* hemostasis. The evidence to support this concept includes comparative genomics that reveal a complete absence of the FXII gene in cetaceans and non-mammalian vertebrates (see Chapter 91). In addition, many mammalian species (e.g. humans, mice, cats, and dogs) with inactivating FXII or PK mutations have no bleeding tendency.³⁷

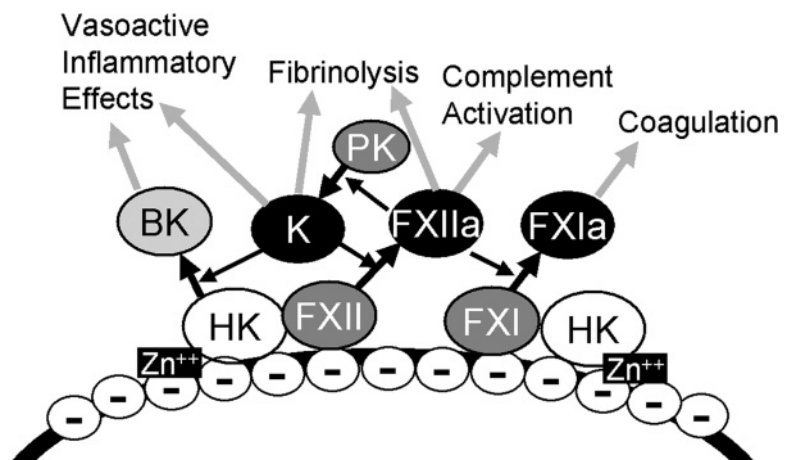
Recent work suggests that the contact pathway plays a role in pathologic thrombus formation.^{16,37} The physiologically relevant negatively charged surface that allows contact activation to influence coagulation *in vivo* has not been identified, although RNA,²² polyphosphate,⁴⁶ and misfolded proteins²⁶ have been proposed to serve this function.

Contact activation has a defined role, however, in several physiologic processes. Contact activation on negatively charged phospholipid cell membranes is primarily responsible for bradykinin generation. The contact pathway has additional anti-adhesive, profibrinolytic, proinflammatory, and antithrombotic properties, and it is involved in angiogenesis (Fig. 84.5).⁷

Factor XII

Factor XII is produced in the liver and circulates as a single chain molecule with molecular weight of about 80 kDa. Cleavage by either kallikrein or plasmin generates a two-chain active enzyme (FXIIa) composed of a light and heavy chain. The heavy chain contains two sites that bind to negatively charged surfaces, and the light chain is a serine protease. Autoactivation to FXIIa may occur when FXII binds to a negatively charged

FIGURE 84.5 The contact pathway. This involves the assembly of FXII and K on a negatively charged surface, resulting in autoactivation of zymogen FXII to the enzyme FXIIa. FXIIa can then activate PK to K, and K can back activate more FXII to FXIIa. K also cleaves HK to generate BK while FXIIa can activate FXI to FXIa. The major products of contact activation have important roles unrelated to coagulation, in inflammation, vasoactive regulation, and fibrinolysis. See Table 84.1 for abbreviations.



surface, especially in the presence of Zn^{2+} . Prekallikrein, HK, and FXI are FXIIa's primary coagulation substrates; however, it also activates the complement pathway and cleaves single chain urokinase plasminogen activator (scuPA) into its two chain form. C1-inhibitor (C1-INH) is the major inhibitor of FXIIa.^{7,44}

Prekallikrein

Prekallikrein is a single chain molecule of about 86 kDa expressed in many tissues, with highest levels in the liver. Approximately 75% of PK circulates bound to HK. Conversion of PK to kallikrein by FXIIa occurs on a negatively charged surface when HK is also bound. This generates a light chain (containing the serine protease) and a heavy chain. Autoactivation of PK at a different cleavage site results in a less active enzyme, β -kallikrein. Kallikrein's primary substrates are FXII and HK. Like FXIIa, kallikrein also cleaves scuPA. Kallikrein is primarily inhibited by C1-INH and α_2 -macroglobulin (α_2 -MG). Kallikrein is also inhibited by protein C inhibitor (PCI) and, when bound to HK, by heparin-AT.⁷

Kininogens

High molecular weight kininogen and low molecular weight kininogen are multi-domain, distinct protein products of the same gene produced by alternative splicing. Both proteins contain an identical heavy chain (domains 1, 2, and 3) and domain 4. The light chain of HK contains domains 5 and 6, while the light chain of its low molecular weight form contains a different domain 5 and no domain 6.

Kininogens have a multitude of functions unrelated to coagulation. Domains 1, 2, and 3, respectively, inhibit atrial natriuretic peptide, prevent calpain-mediated platelet aggregation, and prevent thrombin binding to platelets. Upon cleavage by kallikrein, domain 4 releases the critical vasoactive peptide bradykinin. Domain 5 of HK contains the binding site for charged-surfaces, has anti-adhesive properties affecting protein and cell-binding to neutrophils and endothelial cells, and also influences apoptosis. Domain 6 is the binding site for kallikrein and affects neutrophil activation. Endothelial cells, granulocytes, and platelets contain HK and express binding sites for HK on their resting membrane surface. Platelet activation results in dramatic increase in surface HK, due to release and increased expression of HK binding sites. Zn^{2+} is absolutely required for binding of HK to membrane surfaces.⁷

Extrinsic (Tissue Factor) Pathway

Tissue Factor

Tissue factor, also known as thromboplastin, CD142, and factor III, is a glycosylated single polypeptide chain of approximately 29.5 kDa. It is synthesized in a variety of cells, and expressed on the cell surface as an integral membrane protein. The extracellular portion of the

molecule is responsible for interaction with coagulation proteins. The transmembrane domain anchors TF to the cell surface and is required for procoagulant activity. A small cytosolic domain is dispensable for coagulation functions, but is involved with intracellular signaling in TF's role as a cell-surface receptor.^{34,36}

In physiologic circumstances, TF is expressed by adventitial cells surrounding blood vessels larger than capillaries, differentiating skin keratinocytes, and by other epithelial cells, particularly in mucous membranes and organ capsules. This distribution is consistent with TF's function as a protective "hemostatic envelope" whose role is instant initiation of coagulation. Under pathologic conditions, TF expression can be up-regulated in other cell types by cytokines and other inflammatory mediators. Endothelial cells, vascular smooth muscle cells, monocytes, and granulocytes may express TF in disease states. Tissue factor is found in atherosclerotic plaques and is expressed by some malignant cells. Based on the lack of reports of TF deficiency in humans or animals, and the lack of viability of mice when the TF gene is deleted, TF expression appears to be essential for life.³⁶

Tissue factor serves as the regulatory subunit of the TF-FVIIa enzymatic complex. Cell-surface TF, unlike most cofactors, fully functions as a cofactor without proteolysis. Although FVIIa is a weak enzyme alone, the TF-FVIIa enzyme complex is the most potent activator of coagulation. Consequently, TF-FVIIa is the first enzyme in the clotting cascade.

Tissue factor is primarily sequestered outside the vasculature to prevent intravascular coagulation; however, TF antigen has been detected at low levels in blood. The source and role of this "blood-borne" TF are currently a matter of intense investigation. Blood-borne TF is thought to be associated primarily with microparticles and monocytes. It appears to accumulate in thrombi and may play a role in thrombus propagation. Circulating TF appears to be of limited procoagulant activity under normal circumstances. Some investigators believe that circulating TF is "encrypted" and must be cleaved by an enzyme for activity. Others believe that circulating TF is not fully active because the membrane surface on which it resides is not an appropriate procoagulant membrane.^{34,36}

Factor VII

Factor VII is a glycosylated single chain polypeptide with a molecular weight of approximately 50 kDa. It is a vitamin K-dependent protein produced in the liver and contains 10 Gla residues. It has the shortest half-life of the Gla proteins, so that FVII levels decrease rapidly if the vitamin K cycle or liver function is impaired. Factor VII binding to TF and to membrane surfaces via its Gla domain are calcium-dependent interactions. The FVII zymogen is converted to active FVIIa by proteolysis of a single peptide bond resulting in a two-chain molecule of identical size. Factor VIIa can be generated by a variety of downstream proteases, including FIXa, FXa, FXIIa, FVII activating protease, thrombin, and

plasmin. Factor VII bound to TF can also be autoactivated by TF-FVIIa. The most important physiologic FVIIa activator has yet to be definitively determined.³⁶

Factor VIIa is unique among coagulation enzymes in that some of it circulates with a long plasma half-life of approximately 2 hours (as compared to other serine proteases whose enzyme half-lives are measured in seconds). Approximately 1% of total circulating FVII antigen is active FVIIa, although inter-individual FVIIa levels vary considerably.³⁵ The intriguing question of how and where circulating FVIIa is generated remains unanswered at this time. Factor IXa may be an important contributor of FVIIa generation, as patients deficient in FIX (hemophilia B) have very low levels of FVIIa.⁵⁰ Factor VIIa levels are higher in lymph than plasma, suggesting that some FVIIa may be generated outside the vasculature and returned to the bloodstream via lymphatic circulation.³²

Because some FVII circulates as FVIIa, exposure of blood to TF as a result of injury leads to formation of both TF-FVII complexes and TF-FVIIa complexes. The initial TF-FVIIa formation and autoactivation of TF-FVII to additional TF-FVIIa is believed to generate sufficient enzymatic activity to trigger the clotting cascade via cleavage of the primary TF-FVIIa substrates, FIX and FX.³⁶ The TF-FVIIa complex is often referred to as extrinsic tenase when its target substrate is FX. Deficiency of FVII is associated with a highly variable, but potentially severe bleeding tendency.²⁹

Free plasma FVIIa is not inactivated by any plasma protease inhibitor, hence its long half-life. The TF-FVIIa complex is inhibited by the tissue factor pathway inhibitor (TFPI)-FXa complex, and by heparin-AT (albeit very slowly).³⁶ The inhibition of TF-FVIIa by heparin-AT is unique, in that heparin-AT binding to FVIIa releases a stable FVIIa-AT complex and regenerates fully functional TF. Consequently, FVIIa-AT levels may be an indicator of systemic TF exposure.²⁹

Intrinsic Pathway

The intrinsic cascade primarily occurs on the activated platelet surface. Platelet activation is a critical component of the function of the intrinsic tenase complex (FIXa-FVIIIa). Upon activation, platelets expose many (approximately 6000 per platelet) binding sites for FIXa as well as 1000–2000 each for the cofactor, FVIIIa, and the substrate FX. It is unclear whether these binding sites are simply the negatively-charged membrane surface, or whether a specific protein receptor is also involved. Regardless, co-localization of FIXa, FVIIIa, and FX on the platelet surface increases the rate of FXa generation by many million fold.

Factor XI

Factor XI is an approximately 160kDa protein that is produced in the liver. Its structure is unique among coagulation proteins as a disulfide-linked dimer of two identical polypeptide chains. Factor XI circulates in plasma complexed with HK, which is required for FXI binding to negatively charged surfaces. Prothrombin

can substitute for HK as a cofactor for binding to platelet surfaces.

Factor XI can be activated by FXIIa, FXIa, and thrombin in calcium-dependent reactions. Cleavage results in a 160kDa enzyme with two disulfide linked heavy chains and two active site-containing light chains. Because FXII deficiency does not cause a bleeding tendency, it is likely that thrombin (rather than FXIIa) is the physiologically relevant enzyme generating FXIa. The GP Iba subunit of the platelet GP Ib/IX/V complex binds FXI, localizing it and thrombin on the platelet for efficient cleavage. After activation, FXIa remains surface-bound where it cleaves its substrates, FXI and FIX. Factor IX is FXIa's preferred substrate.

Inhibition of FXIa depends on its localization. Plasma inhibitors include its major inhibitor, α_1 protease inhibitor (α_1 -PI), and C1-INH, α_2 -antiplasmin (α_2 -AP), AT, protease nexin 1, plasminogen activator inhibitor 1 (PAI-1), and PCI. Platelet-bound FXIa is protected from α_1 -PI inactivation. When bound to the endothelial cell surface, protease nexin 2, in concert with surface heparan sulfate proteoglycans (HSPGs), strongly inhibits FXIa. This differential inhibition may serve to restrict FXIa mediated cleavage of FIX to the platelet surface.

Factor IX

Factor IX's structure and size (about 55kDa) are similar to those of FVII. Factor IX is a glycosylated single-chain polypeptide, produced in the liver. Post-translational carboxylation of 12 residues by the vitamin K cycle is required for normal Gla domain function and consequently for full-membrane binding capability.²

Tissue factor-FVIIa or FXIa activate FIX bound (in the presence of calcium) to a procoagulant membrane surface. Cleavage of two FIX sites releases a 35-residue activation peptide, resulting in a two-chain enzyme of about 45kDa. The catalytic domain is in the heavy chain portion of the molecule. The presence of detectable levels of the FIX activation peptide in blood suggests that coagulation is ongoing, but controlled by inhibitory pathways.²

The primary substrate for FIXa is FX. The FIXa-FVIIIa complex on a suitable membrane surface (usually the platelet surface) in the presence of calcium is a powerful generator of FXa. Either FIX or FIXa may bind to endothelial cells, via specific receptors (collagen IV). Binding to the endothelial surface supports activation of FX by FIXa-FVIIIa in the absence of platelets. Factor IXa alone (without its cofactor FVIIIa) is able to activate FVII in the absence of TF.

FIXa is primarily inhibited by AT-HSPG, although at a relatively slow rate compared to AT's inhibition of FXa or thrombin.

Factor VIII

Factor VIII synthesis and secretion are extremely complex. Two distinct interactions involving metal ions (copper and calcium/manganese) are vital for normal FVIII structure and function. The primary site of FVIII

production is unclear, although the liver and the reticuloendothelial system are strongly implicated. Factor VIII is synthesized as a single chain protein containing three A domains, a B domain, and two C domains, with multiple free cysteine residues and disulfide bonds. After translation, FVIII is translocated to the endothelial reticulum (ER), for modification. Factor VIII is retained within the ER by two protein chaperone systems, immunoglobulin-binding protein and calnexin/calreticulin. Improperly folded FVIII is extracted from the ER and degraded. Factor VIII then transits to the Golgi apparatus for further processing, which includes cleavage at two sites. The resulting protein consists of a heavy chain (160 or 200 kDa) and a light chain (80 kDa) which are not connected to one another unless von Willebrand factor (VWF) is also bound.²³

Binding to VWF enables proper association of the two FVIII chains and markedly improves its intracellular and plasma stability. The ratio of the two proteins is maintained at one molecule of FVIII to 50–100 subunits of VWF. Although somewhat species-dependent, VWF deficiency is associated with lower levels of circulating FVIII, and infusion of desamino-8-D-arginine vasopressin (DDAVP) to increase circulating VWF levels elicits a concomitant increase in FVIII. Some FVIII is stored with VWF in storage granules, such as Weibel-Palade bodies of endothelial cells and α granules of platelets.

Factor VIII can be dissociated from VWF by exposure to negatively-charged phospholipids. Thrombin cleavage of FVIII at multiple sites activates FVIII to FVIIIa and releases it from VWF. Factor VIII can be activated by FXa, although the maximal activation by thrombin is greater than FXa. Factor VIIIa interacts with P_{Ser} on membrane surfaces by stereo-selective recognition of the P_{Ser} head group via a phospholipid-binding site in the light chain. Factor VIIIa also interacts with two platelet receptors, GP Ib and $\alpha_{IIb}\beta_3$. Platelet binding of FVIII is inhibited by VWF. The FIXa-FVIIIa enzyme/cofactor complex assembles in a calcium-dependent manner on procoagulant phospholipid surfaces, and is referred to as intrinsic tenase, because its target substrate is FX.

Factor VIIIa is inherently labile due to spontaneous dissociation of its subunits; however, FIXa binding stabilizes FVIIIa and delays decay. Factor VIIIa is cleared from the circulation by low density lipoprotein receptor-related protein (LRP), a common cell-surface receptor found on a variety of cell types. Proteolytic cleavage by activated protein C (APC) and FXa also inactivates FVIIIa.²³

Common Pathway

Factor X

Factor X is a Gla protein of approximately 59 kDa. It is a glycosylated single-chain polypeptide requiring post-translational carboxylation of 11 glutamic acid residues. Factor X is activated to FXa by either extrinsic tenase (TF-FVIIa) or intrinsic tenase (FIXa-FVIIIa) on a suitable membrane surface in the presence of calcium. The acti-

vation is due to a single cleavage site and results in a 46 kDa enzyme.

Factor Xa forms the prothrombinase complex with its cofactor FVa. Factor Xa is strongly inhibited by AT-HSPG and by TFPI. It is also inhibited by the enzyme/cofactor protein Z-dependent protease inhibitor (ZPI)/PZ. Inhibition by ZPI/PZ is calcium and phospholipid dependent.

Factor V

Factor V circulates in plasma as a glycoprotein of approximately 300 kDa. Portions of the molecule are very similar in structure to FVIII. Like FVIII, FV contains a copper ion, and requires post-translational modifications and molecular chaperones for successful cellular trafficking. Approximately 20% of FV in blood is contained within platelet α granules. The plasma half-life of FV in man is fairly long (approximately 13 hours).²¹

Factor V is activated to FVa by cleavage at two sites that releases a large activation peptide, and a resultant two-chain cofactor of about 160 kDa. Activation is primarily due to thrombin cleavage, but FXa can also activate FV when both are bound to a procoagulant membrane surface. Activation by FXa is less efficient than activation by thrombin; however, FV released from platelets is somewhat more susceptible to FXa activation than plasma FV.²¹

Factor Xa and FVa form an enzyme/cofactor complex, referred to as the prothrombinase complex, in the presence of calcium on P_{Ser}-containing membrane surfaces. While FVa binding to the membrane is not calcium dependent, the binding of FX and prothrombin to the membrane is calcium dependent, but of low affinity. Binding of FVa to a negatively charged membrane surface markedly increases the affinity of FXa for FVa, and enhances formation of the complex. Platelets and microparticles primarily provide this membrane surface *in vivo*.²¹

Proteolysis of FVa by APC results in its inability to bind prothrombin, and therefore loss of function of the prothrombinase complex. Inactivation of FVa by APC occurs more rapidly on endothelial cells than on activated platelet surfaces.^{12,21}

Prothrombin

Prothrombin is a 72 kDa glycoprotein synthesized in the liver that (like other Gla proteins), undergoes vitamin K-dependent post-translational carboxylation of 10 glutamic acid residues. Prothrombin is the second most abundant coagulation protein in plasma, after fibrinogen.²⁰

In addition to the Gla domain, prothrombin contains two kringle domains and a serine protease precursor domain. Prothrombin cleavage at two distinct sites by prothrombinase generates its most abundant active form, α thrombin. The first *in vivo* cleavage site is generally between the A and B chains of its serine protease domain to form the important intermediate, meizothrombin. Meizothrombin contains all the domains of

the prothrombin molecule, with a fully active enzyme site, but it is less active toward fibrinogen and platelets than α thrombin. After the second cleavage, prothrombin fragments 1 (containing the Gla domain and kringle 1) and 2 (containing kringle 2 domain) are removed. The A and B chains of the much smaller (36.7 kDa) α -thrombin molecule remain. Under some conditions the order of cleavage is reversed, releasing the membrane binding Gla domain prior to activation of the protease.²⁰

Coagulation Inhibitors

Tissue Factor Pathway Inhibitor

Tissue factor pathway inhibitor is the key regulator of the extrinsic pathway. It is a Kunitz type inhibitor synthesized by endothelial cells and expressed on their surface. Alternative splicing creates two forms: TFPI α and TFPI β . Both forms are bound to the cell surface: TFPI α via a not yet identified glycosylphosphatidylinositol (GPI)-linked protein, and TFPI β via interactions between the C terminal region and an identified GPI anchor.⁴¹ Cell surface associated heparan sulfates also play a role in the binding of TFPI to the endothelial cell.^{1,30}

The majority (80–85%) of TFPI is attached to the endothelial cell surface. A small amount (10%) of TFPI circulates in plasma associated with lipoproteins, and platelet α granules also contain TFPI.¹ Intravenous heparin administration causes release of TFPI from the endothelial cell-surface and a marked increase in plasma TFPI.³⁰ Free TFPI is cleared from the circulation primarily by the liver and kidneys.

Both TF-FVIIa and FXa are susceptible to TFPI inhibition. In the absence of FXa, TFPI is only a weak inhibitor of TF-FVIIa. The second Kunitz domain of TFPI binds to FXa; however, the FXa-FVa complex is protected from inhibition by TFPI, particularly if the substrate prothrombin also binds.³¹ Recent work indicates that PS serves as a TFPI cofactor, enhancing its inhibitory function,⁴³ and that polyphosphate released from platelet dense granules profoundly abrogates TFPI inhibition.⁴⁶

The TFPI-FXa complex is a strong inhibitor of TF-FVIIa, binding via the first Kunitz domain. Endocytosis and degradation of the quarternary complex (TFPI-FXa-TF-FVIIa) is mediated by cell-surface associated LRP.¹

Inherited deficiency of TFPI has not been described, suggesting that complete lack of this inhibitor is incompatible with life.

C1-Inhibitor

C1-Inhibitor is the largest member of the serpin family. It has an unusual structure for this class of proteins, containing a typical serpin domain and an additional N-terminal domain of ill-defined structure and function. It is a relatively slow inhibitor of its target proteases,

but C1-INH inhibition is potentiated by glycosaminoglycans such as heparin and heparan sulfates.⁴⁹

The C1-INH is the major inhibitor of the contact pathway, acting on FXIIa and kallikrein, and the downstream protease FXIa. Hereditary deficiency of C1-INH has no impact on hemostasis. Rather, it causes excess episodic production of bradykinin, producing the disease phenotype hereditary angioedema.⁴⁹

Antithrombin

Antithrombin is the prototype serpin; however, it is a relatively inefficient inhibitor of its target enzymes. The “bait” portion of the AT molecule is available only if AT binds to its cofactor. This cofactor is a specific pentasaccharide sequence that is found on approximately 30% of pharmaceutical heparin molecules and on endothelial-bound HSPG molecules. Upon AT-pentasaccharide binding, the bait loop becomes available as a target for the enzyme to be inhibited. Antithrombin’s inhibitory activity is enhanced up to several thousand-fold by interaction with heparins, but the impact varies with heparin structure and the target protease. Pentasaccharide alone is sufficient for AT inhibition of some enzymes, while longer heparin molecules are required for inhibition of others. In particular, AT inhibition of thrombin requires, in addition to the specific pentasaccharide sequence, heparin molecules of at least 18 sugar units to interact with thrombin.

Antithrombin molecules in circulation have limited inhibitory capabilities in the absence of the necessary pentasaccharide. Endogenous activation of AT occurs when it binds to heparans on the surface of the endothelial cell. Additionally, endothelial cell surface thrombomodulin (TM) can also bind AT, which enhances AT inhibition of thrombin approximately 8-fold.^{4,48}

In addition to inhibiting thrombin, AT-heparin has broad inhibitory activity against a variety of serine proteases. These include FXIIa, FXIa, kallikrein, FIXa, and FVIIa. The primary targets are FXa and thrombin. Platelet-bound thrombin (via GP Ib α) and thrombin bound to the fibrin clot or fibrin degradation products are refractory to AT-heparin inhibition. In contrast, inhibition of FVIIa requires that the enzyme be bound to its cofactor (TF) on a cell surface, and kallikrein is more readily inhibited when bound to HK on a cell surface.

Antithrombin bound to a target protease forms a stable complex with long half-life. The complexes are primarily cleared in the liver by LRP. Inherited or acquired AT deficiency is associated with development of pathologic thrombosis.⁴

Protein C Pathway

The PC pathway involves a complex interplay among endothelial cell-surface molecules, thrombin, PC, and PS. Activated PC is an essential regulator of thrombin generation due to its inactivation of the critical cofactors, FVa and FVIIIa. The thrombotic disorders in people attributed to inherited and acquired PC deficiency demonstrate its important anticoagulant action.

Protein C is an approximately 62kDa vitamin K-dependent protein, synthesized in the liver, containing a Gla domain with nine glutamic acid residues. It circulates as a two-chain zymogen that is activated by thrombin (or meizothrombin) to APC. Cleavage at two sites results in removal of a 12-residue activation peptide and formation of an approximately 56kDa two-chain enzyme, APC. Plasmin activation also produces limited amounts of APC. As a Gla containing protein, PC activation is calcium dependent and markedly enhanced by negatively charged phospholipid surfaces, in particular phosphatidylethanolamine (PEth). Thrombin alone is a poor activator of PC unless it is bound to TM.¹²

Thrombomodulin is an endothelial transmembrane molecule that interacts with thrombin to influence its substrate specificity. Thrombomodulin inhibits thrombin's interaction with fibrinogen and protease activated receptors, and markedly enhances the ability of thrombin to cleave PC. Cellular expression of TM is modulated by a large number of factors. It is up-regulated in response to cyclic AMP, thrombin, vascular endothelial growth factor, and platelet factor 4. It is down-regulated by inflammatory mediators (e.g. tumor necrosis factor, interleukin 1, endotoxin), hypoxia, transforming growth factor β , and neutrophil activation.¹²

Protein S is an approximately 69kDa vitamin K dependent protein with a Gla domain that interacts with membranes. Protein S is the cofactor for APC mediated cleavage of FVa and FVIIIa, and for TFPI's inhibition of FXa.⁴³ Approximately 60% of circulating PS is bound to a large (540kDa) member of the complement system, C4 binding protein (C4BP). C4 binding protein is an acute phase reactant and therefore increases in inflammatory conditions. Protein S complexed with C4BP is unable to provide APC cofactor activity. Activated platelets and neutrophils contain proteases that cleave and inactivate PS.^{11,12}

Endothelial protein C receptor (EPCR) is a transmembrane protein found primarily on arterial endothelium. The EPCR can substitute for negatively-charged phospholipids in promoting activation of PC. It binds directly to PC via the Gla domain, anchoring it to the cell surface. The APC-EPCR complex is unable to bind PS and does not have anticoagulant activity, but may interact with other receptors, potentially contributing to the anti-inflammatory effects of APC.^{12,13} Once APC is produced and dissociates from thrombin-TM or EPCR, it assembles with PS on a procoagulant membrane surface and rapidly inactivates FVa and FVIIIa. A mutation at one of the APC cleavage sites on FV ("FV Leiden") confers resistance to APC inactivation, and is a common hereditary prothrombotic disorder in people. Activated PC cleaves FVa more efficiently on endothelial surfaces than on platelets,³⁸ and FVIIIa is resistant to APC inactivation when bound to VWF. The anticoagulant activity of APC is highly species-specific.¹²

The PC pathway appears to have a major role in linking inflammation and coagulation, in part due to the ability of APC to down-regulate thrombin generation. Additionally, APC alters gene expression

in endothelial cells via activation of membrane surface protease activated receptors (PARs), resulting in anti-apoptotic and anti-inflammatory effects. Activated PC-PS also inactivates an important inhibitor of fibrinolysis (PAI-1).¹² Activated PC has a relatively long half-life of about 15 minutes. It is inhibited by α_1 -PI, PCI, and α_2 -MG.

Miscellaneous Inhibitors

Heparin cofactor II (HC) is a 66kDa serpin with some similarities to AT. It requires binding to proteoglycans for full function, but the mechanism differs from that of AT. Heparin cofactor II is strongly activated by binding to dermatan sulfate and has a narrow spectrum of activity restricted to thrombin inhibition. Although HC-thrombin complexes are detectable in vivo, HC deficiency is not associated with increased risk of thrombosis. Roles for HC in inflammation and wound healing, rather than in regulation of coagulation, have been suggested.⁴

Protein Z-dependent protease inhibitor is a 72kDa serpin that strongly inhibits FXa when bound with its cofactor, PZ, on a phospholipid surface in the presence of calcium. Protein Z is a 62kDa vitamin K-dependent protein that binds to the membrane via its Gla domain. Thrombomodulin also acts as a cofactor for ZPI. Factor XIa is also susceptible to ZPI inactivation, in a heparin-dependent reaction that does not require calcium or phospholipids.

α_1 -Protease inhibitor, PCI, and α_2 -MG are inhibitors that act on a variety of serine proteases in coagulation and other systems. α_1 -Protease inhibitor is a 52kDa molecule, synthesized primarily in the liver, whose major physiologic role is the protection of alveolar tissue from proteolytic damage by enzymes like neutrophil elastase. It is an acute phase reactant, and is up-regulated by inflammatory mediators.¹⁴

Protein C inhibitor is a 53kDa serpin that inhibits APC, FXa, FXIa, kallikrein, thrombin, thrombin-TM, urokinase plasminogen activator (uPA), and tissue plasminogen activator (tPA). Its inhibitory activity is enhanced by heparin and it is involved in modulating vascular permeability, tissue regeneration, proteolysis in the kidney, and tumor cell invasion.⁴⁷

α_2 -Macroglobulin is a large, 725kDa inhibitor of matrix metalloproteinases that works through a unique mechanism. The target enzyme cleaves an α_2 -MG "bait" region to produce a conformational change that provides sites for covalent enzyme attachment. The enzyme binds and is entrapped by the inhibitor, with resultant α_2 -MG-enzyme complexes internalized and degraded via LRP binding.

CELLS THAT PARTICIPATE IN HEMOSTASIS

Endothelial Cells

The endothelium, once considered an inactive barrier between the blood and the subendothelial tissues, is now recognized as a highly active organ. The endothe-

lium has a variety of functions including regulation of coagulation and fibrinolysis, local control of vascular tone, systemic regulation of blood pressure, interaction with lipoprotein metabolism, production of angiotensin converting enzyme, presentation of histocompatibility antigens, and interaction with leukocytes. The majority of endothelial cells are in capillary beds due to the surface area distribution of the vascular system, and most interactions between the endothelium and flowing blood occur in capillaries where the ratio of endothelial cell surface to blood volume is high.

Antithrombotic Properties of Endothelium

The prevention of coagulation in flowing blood is normal endothelium's primary role in hemostasis (Fig. 84.6).

Non-activated, resting endothelial cells have anticoagulant properties, in part due to proteins expressed on their surface and via a neutral membrane charge that is incapable of supporting coagulation reactions. Heparan sulfate proteoglycans are glycosaminoglycans expressed by endothelial cells on the luminal surface, in contact with flowing blood. Much larger abluminal stores of HSPG act as a reservoir. Antithrombin bound to HSPGs (at pentasaccharide sequences) is then able to inactivate its serine protease targets (e.g. thrombin, FXa). Resting endothelial cells also express TM. Thrombin, once bound to TM, demonstrates anticoagulant rather than procoagulant properties. Thrombomodulin-bound thrombin is unable to cleave fibrinogen or activate PARs, and the TM-thrombin complex rapidly activates PC. Expression of TM is 100-fold higher in capillary endothelium compared to endothelium in the major vessels. Thrombin circulating in large vessels is therefore quickly extracted when the blood passes through capillaries. Lastly, TFPI on the endothelial cell surface prevents thrombin generation by acting as an upstream inhibitor of FXa and TF-FVIIa.

Prothrombotic Properties of Endothelium

Endothelial cells store VWF in granules called Weibel-Palade Bodies. This protein has important roles in FVIII biology, and in mediating platelet adhesion and aggregation (Chapter 81). Endothelial cells express a specific G protein coupled receptor for thrombin. Thrombin binding to this receptor initiates a number of procoagulant and proinflammatory events. Intracellular signaling initiates endothelial secretion of VWF and P-selectin from Weibel-Palade bodies, nitric oxide (NO) release, and phospholipase A₂ activation that results in platelet activating factor (PAF) production. Activated endothelial cells express intracellular adhesion molecule 1, generate interleukin 1, and secrete both plasminogen activators and PAI-1.

Other Properties of Endothelial Cells

Endothelial cells synthesize NO in response to a variety of stimuli including thrombin, histamine, ATP, bradykinin, and acetylcholine. Nitric oxide inhibits platelet activation and causes vasodilation. Thrombin and other stimuli also induce endothelial cell synthesis of prostacyclin (PGI₂), a platelet inhibitor that also maintains vascular relaxation.⁸

Platelets

Platelets are a fundamental requirement for normal hemostasis. Activated platelets adhere to the site of injury, secrete molecules necessary for coagulation and for wound healing, and form platelet aggregates that obstruct the site of injury, preventing further blood loss. Platelets provide the primary membrane surface for thrombin generation, allowing for formation of a platelet-fibrin clot (Chapter 76).

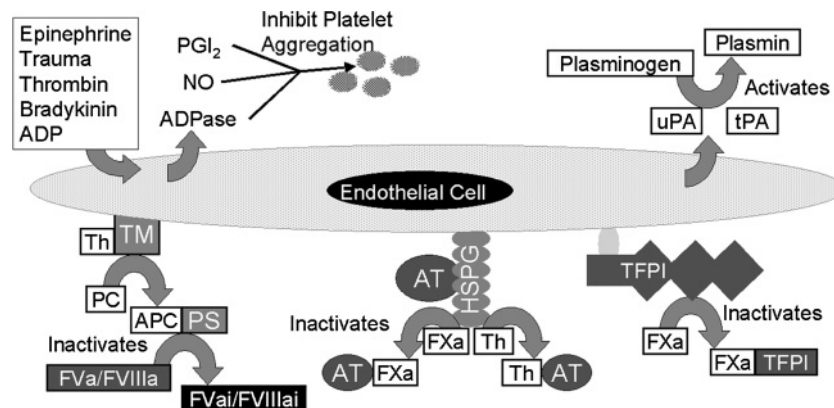


FIGURE 84.6 Anticoagulant properties of endothelial cells. Endothelial cells are thromboresistant under normal physiologic conditions. They express adenosine diphosphatase (ADPase) and synthesize and release prostacyclin (PGI₂) and nitric oxide (NO), leading to inhibition of platelet aggregation. They also produce urokinase (uPA) and tissue-type (tPA) plasminogen activators which activate plasminogen to plasmin, the enzyme that lyses fibrin clots. Endothelial cells express thrombomodulin (TM), heparan sulfate proteoglycans (HSPG) and tissue factor pathway inhibitor (TFPI) on their membrane surface, which collectively inhibit generation of thrombin (Th). TM acts as a cofactor for Th-mediated activation of the protein C (PC) pathway; HSPG acts as a cofactor for antithrombin (AT) inhibition of FXa; TFPI inhibits FXa and TF-FVIIa.

Tissue Factor Bearing Cells

As previously discussed, exposure of TF on a TF-bearing cell is the physiologically relevant trigger for *in vivo* coagulation. Tissue factor-bearing cells are primarily outside the vasculature, particularly in the adventitia, skin, mucous membranes, and organ capsules. Under pathologic conditions leukocytes, endothelial cells, and neoplastic cells may also express TF.

Microparticles

Microparticles (MPs) are intact vesicles surrounded by membranes. They range in size from 2–20% of the size of a red blood cell (i.e. 0.1–1 μ m diameter) and arise when activated or apoptotic cells shed bits of membrane. Cytokines (such as tumor necrosis factor and interleukin-6), thrombin, shear stress, and hypoxia can stimulate MP formation. Under normal conditions, MPs are primarily derived from endothelial cells, platelets, and monocytes, but in certain disease states, MPs may arise from granulocytes and erythrocytes. The quantity of circulating MPs is increased in certain illnesses such as diabetes mellitus, sepsis, and cardiovascular disease and may contribute to pathologic coagulation in a variety of disorders.³³

Microparticles contain cell surface proteins similar to those found on their parent cell (e.g. ultra-large VWF monomers on endothelial cell-derived MPs, TF on monocyte-derived MPs) that can participate in coagulation reactions, especially when the MP expresses a procoagulant surface. The contribution of MPs to normal hemostasis is currently under active investigation.^{24,33,40}

The Role of Cell Surface Membranes

All cells are surrounded by a lipid membrane bilayer which contains a large number of membrane surface proteins. In most mammalian cells, membrane bilayers consist primarily of cholesterol and phospholipids with neutral head-groups such as phosphatidylcholine (PChol), sphingomyelin, sugar-linked sphingolipids and PEth. The membranes also contain phospholipids with negatively charged-head groups, primarily PSer. In the resting state, PEth and PSer are sequestered on the membrane's inner surface. This membrane asymmetry is essential and tightly controlled by lipid transporters. Flippase actively transports PSer from the external to the internal leaflet while floppase transports PChol in the opposite direction. These ATP-dependent enzymes maintain cell membrane asymmetry in the resting state.

Cell-injury or activation stimuli that produce a sustained increase in intracellular calcium induce a poorly characterized "scramblase" that shuffles phospholipids between the membrane leaflets. This results in the appearance of PSer and PEth on the external membrane surface.^{40,51} Although the mechanisms of coagulation on activated membrane surfaces are not fully understood, it is known that membrane-surface PSer markedly (often by a factor of thousands to hundreds of thousands) increases the rate of coagulation reactions. The

presence of PEth enhances the properties of PSer-expressing membranes, so that less PSer is required for maximum speed when PEth is present. It has been proposed that PEth aids in grouping PSer into clusters that support preferential binding of coagulation proteins.^{36,53} Under normal physiologic conditions, cells do not express these procoagulant phospholipids on their outer membrane. As a consequence, thrombin production is limited to cell surfaces in an injured area that have been triggered to externalize PSer (and PEth). The ability of cells to control the composition of their membrane surface constitutes a powerful method of localizing coagulation reactions.⁵³

CELL BASED MODEL OF THROMBIN GENERATION

Our new understanding of hemostasis incorporates the role of cells. This model suggests that *in vivo* coagulation occurs in distinct overlapping phases that require the participation of two different cell types: TF-bearing cells and platelets.

Initiation

All evidence to date indicates that TF is the sole relevant initiator of coagulation *in vivo*. Cells expressing TF are generally localized outside the vasculature, which prevents initiation of coagulation under normal flow circumstances with an intact endothelium. Some circulating cells (e.g. monocytes) and MPs may express TF on their membrane surface, but this TF under normal conditions is thought to be inactive or "encrypted".^{27,36}

Upon injury, flowing blood is exposed to a TF-bearing cell and FVIIa rapidly binds to TF.^{35,36} The TF-FVIIa complex then activates additional TF-FVII to TF-FVIIa, which generates small amounts of FIXa and FXa. Although it occurs slowly, FV can be activated directly by FXa. The resultant enzyme and cofactor (FXa, FVa) form the prothrombinase complex, which subsequently cleaves prothrombin to generate a small amount of thrombin on the surface of TF-bearing cells. Any FXa that dissociates from the membrane surface is rapidly inactivated by either TFPI or AT-HSPG. Factor Xa, therefore, is effectively restricted to the surface of the TF-bearing cell on which it was generated. However, FIXa is not inhibited by TFPI, and much more slowly inhibited by AT than FXa. Consequently, trace amounts of generated FIXa can dissociate and move to the surface of nearby platelets or other cells.^{19,42}

Since TF is always expressed in the perivascular space, any FVIIa that leaves the vasculature will bind to TF and potentially initiate coagulation. The gaps in the physiologic endothelial envelope under normal conditions are very small. Most of the upstream coagulation proteins are relatively small, whereas some of the downstream proteins are much larger. These downstream substrates, as well as platelets, are sequestered from the extravascular space. Coagulation progresses beyond initiation (and its generation of trace amounts

of thrombin) only when the injury allows platelets and larger proteins to leave the vascular space and adhere to extravascular TF-bearing cells (Fig. 84.7A).^{19,42}

Amplification

Once a small amount of thrombin has been generated in the initiation phase, it is available to activate platelets that have leaked from the vasculature at the site of injury. Thrombin binding to platelet surface receptors causes extreme changes in the surface of the platelet, resulting in shape change, shuffling of membrane phospholipids to create a procoagulant membrane surface, and release of granule contents that provide additional “fuel for the fire.” Platelet granules contain a large number of proteins and other substances which include raw materials for clotting reactions, agonists to induce further platelet activation, and calcium. Calcium may induce membrane externalization and clustering of P_{Ser}, and promotes binding of coagulation proteins to the activated membrane surface. In addition to activating platelets, thrombin generated in the initiation phase cleaves FXI to FXIa and activates FV to FVa on the platelet surface. Thrombin also cleaves VWF from FVIII and activates FVIII to FVIIIa (Fig. 84.7B).^{19,42}

Propagation

The release of platelet granule contents recruits additional platelets to the site of injury. The propagation phase occurs on the surface of these platelets. Expression of ligands on their surface results in cell-cell interactions that lead to platelet aggregation. Factor IXa generated by TF-FVIIa in the initiation phase can bind to FVIIIa (generated in the amplification phase) on the platelet surface. Additional FIXa is generated due to cleavage of FIX by FXIa (generated during amplification on the platelet surface). Once the intrinsic tenase complex forms (FIXa-FVIIIa) on the activated platelet surface, it rapidly begins to generate FXa on the platelet. Since FXa generated on TF-bearing cells is rapidly inhibited if it moves away from the cell surface, it cannot easily reach the platelet surface. The majority of FXa in the propagation phase, therefore, must be generated directly on the platelet surface by the intrinsic tenase complex. This generated FXa then rapidly binds to FVa (generated by thrombin in the amplification phase) and cleaves prothrombin to thrombin. This prothrombinase activity results in a burst of thrombin. When enough thrombin is generated with enough speed to cleave fibrinogen, a clot forms (Fig. 84.7C).^{19,42}

THROMBIN

Thrombin is generated from prothrombin by cleavage at two activation sites. Thrombin is the terminal coagulation protease responsible for fibrin formation. Thrombin generation, however, is not the end of coagulation. Many steps vital to normal hemostasis occur temporally *after* generation of a fibrin gel. Approximately 5% of the

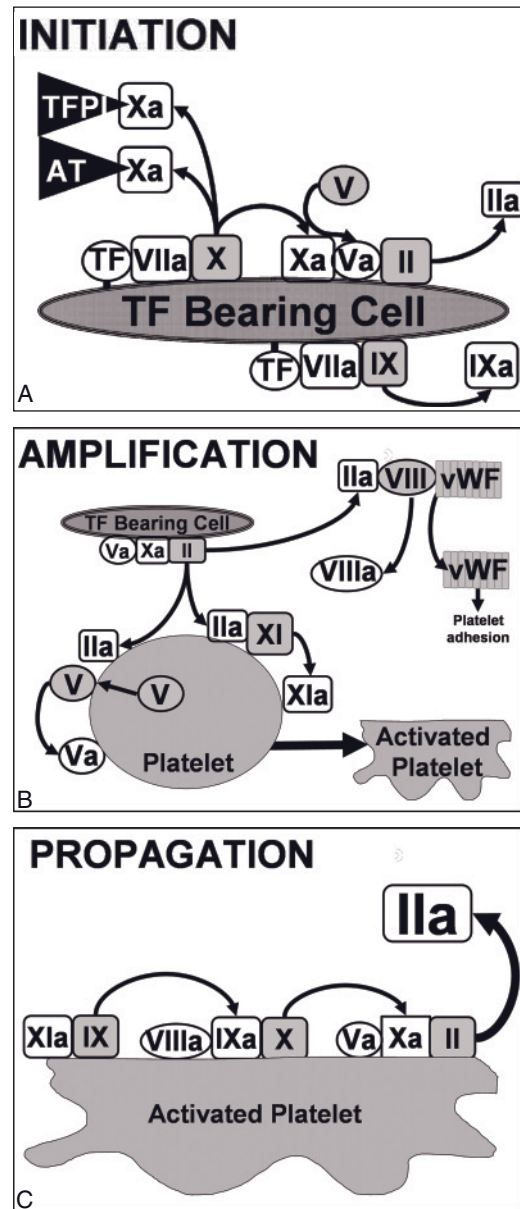


FIGURE 84.7 Cell-based model of fibrin formation. In this model thrombin generation occurs in overlapping phases. (A) Initiation phase: This phase occurs on the TF-bearing cell and is initiated when injury exposes the TF-bearing cell to the flowing blood. It results in the generation of a small amount of FIXa and thrombin that diffuse away from the surface of the TF-bearing cell to the platelet. (B) Amplification phase: In the second phase, the small amount of thrombin generated on the TF-bearing cell activates platelets, releases VWF and leads to generation of activated forms of FV, FVIII, and FXI. (C) Propagation phase. In the third phase the various enzymes generated in earlier phases assemble on the procoagulant membrane surface of the activated platelet to form intrinsic tenase, resulting in FXa generation directly on the platelet surface. Prothrombinase complex forms and results in a burst of thrombin generation directly on the platelet. See Table 84.1 for abbreviations. (Reproduced from Smith S, The cell-based coagulation model. *J Vet Emerg Crit Care* 19:3–10, with permission.)

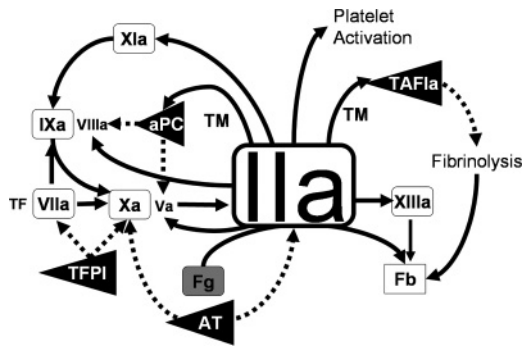


FIGURE 84.8 Central role of thrombin. Thrombin plays a central role in hemostasis, acting as both a procoagulant and an anticoagulant. Procoagulant thrombin is generated in small quantities during the initiation phase (which occurs on the TF-bearing cell), then in larger quantities on the platelet surface in the propagation phase. Thrombin is responsible for platelet activation which provides the procoagulant surface needed for the burst of thrombin generation during propagation. Thrombin cleaves fibrinogen to fibrin, resulting in clot formation. Thrombin also impacts clot structure through activation of cross-linker FXIII, and fibrinolysis inhibitor TAFI. Thrombin becomes anticoagulant via thrombomodulin-mediated activation of the protein C system. See Table 84.1 for abbreviations. (Reproduced from Smith S, The cell-based coagulation model. *J Vet Emerg Crit Care* 19:3–10, with permission.)

prothrombin that will be cleaved has been converted to thrombin at the time of fibrin gel formation, as detected in routine plasma-based clotting assays.²⁸

Free thrombin has an extremely short plasma half-life of less than 15 seconds due to rapid inhibition by AT-HSPG.¹⁰ Thrombin bound to the fibrin clot, however, is both enzymatically active and protected from inhibition. In addition to its function in cleavage of fibrinogen to fibrin, thrombin has many additional roles in coagulation and fibrinolysis, as well as functions in cell regulation (Fig. 84.8).²⁵

Feedback Regulation of Coagulation

Thrombin provides positive feedback to the process of coagulation by back-activating a number of upstream proteases and cofactors. These include FVII, FXI, FVIII, and FV. Thrombin bound to endothelial surface TM acts primarily to inhibit coagulation via activation of the PC anticoagulant pathways. Moreover, TM-binding inhibits the interactions between thrombin and fibrinogen, limits thrombin activation of platelet PARs, and promotes activation of the fibrinolytic mediators, TAFI and scuPA. Through these feedback mechanisms, thrombin influences the quantity and speed of its own generation, and the amount of fibrin deposition and degradation.

Platelet Activation

Thrombin, along with many other serine proteases, can bind and cleave PARs. These cell-surface associated G-coupled proteins are found on platelets, endothelial cells, smooth muscle cells, leukocytes, and other cell

types. Cleavage by thrombin untethers a portion of the PAR molecule that self inserts into the receptor, thereby initiating intracellular signalling. This PAR-mediated signaling is the major mechanism by which thrombin activates platelets. Thrombin also binds to the platelet GP Ib/IX/V complex via a binding site in GP Ib α , although how this binding mediates platelet activation is not clearly understood.¹⁰

Fibrin Formation

The primary function of thrombin is conversion of fibrinogen to fibrin. Thrombin cleaves two short peptides from the fibrinogen molecule, fibrinopeptide A (FpA) and fibrinopeptide B (FpB), thus exposing binding sites that interact with pre-existing sites on other fibrin molecules. The interaction between multiple fibrin molecules results in spontaneous polymerization into an insoluble fibrin gel.

Activation of Factor XIII

Factor XIII (FXIII) is a 320 kDa protein that consists of five chains arising from transcription of two different genes. Half of the circulating pool of functional FXIII is free in plasma, and the remainder is released from platelet α granules. Factor XIII cleavage by thrombin releases an activation peptide, resulting in a 312 kDa active enzyme. Thrombin cleavage of free FXIII is slow; however, polymerized fibrin acts as a cofactor, markedly increasing the rate of FXIIIa generation. Consequently, FXIIIa is generated primarily after a critical weight of fibrin has polymerized.

Factor XIIIa is a transglutaminase whose primary function is crosslinkage of fibrin fibrils. This activity is calcium dependent, and critical for normal clot strength and stability. Factor XIIIa also crosslinks fibronectin to fibrin and to collagen, which influences anchoring of the clot to the vessel wall. Additionally, FXIIIa has roles in cell adhesion, angiogenesis, and tissue repair.

FIBRINOGEN AND FIBRIN CLOT STRUCTURE

Fibrinogen is a soluble 340 kDa dimer consisting of three pairs of disulfide-bridge linked polypeptides. It is the most abundant coagulation protein in plasma and is abundant in platelet α granules. In addition to the role of fibrinogen as a precursor to fibrin, fibrinogen mediates intracellular interactions between platelets in conjunction with thrombospondin (Chapter 76).

Thrombin cleaves fibrinogen at two sites, releasing FpA and FpB. Upon FpA release, soluble fibrin monomer spontaneously assembles into an insoluble dimer. This polymerization occurs because removal of FpA exposes a binding site “A knob” that interacts with a complementary binding “hole” on separate fibrin molecules. Initial polymerization produces a dimer with a half-staggered structure. Dimers continue to associate to form longer, two-fibrin-wide strands, called protofibrils.^{3,18} Subsequent removal of FpB allows for lateral

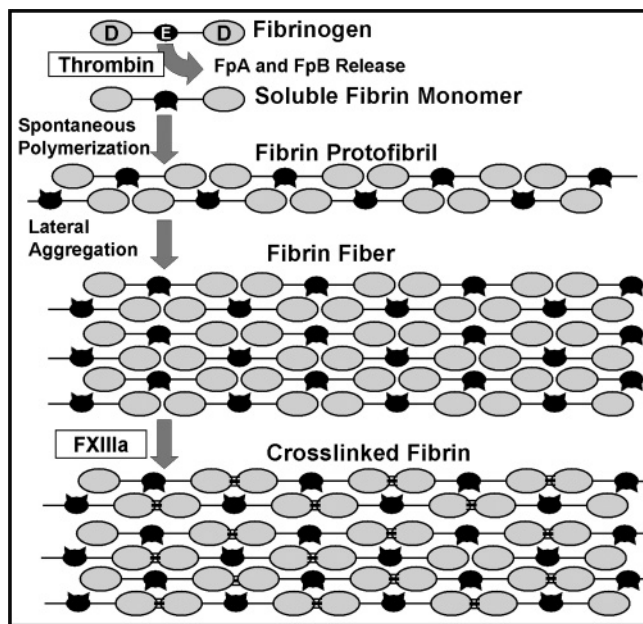


FIGURE 84.9 Fibrin formation. Fibrinogen consists of a central E domain connected by coiled chains to two lateral D domains. Thrombin cleaves fibrinogen to fibrin monomer by removing two short peptides from the E domain (fibrinopeptides A and B) exposing binding sites in the E domain. These exposed sites then interact with corresponding binding sites on the D domains of other fibrin molecules, resulting in spontaneous polymerization into a fibrin protofibril. Multiple protofibrils laterally polymerize, forming a fibrin fiber. FXIII is activated to FXIIIa by thrombin. FXIIIa creates cross-links between D domains of contiguous fibrin monomers.

aggregation of protofibrils of increasing width via the “B knob” interacting with its complementary binding site. Protofibrils are stabilized by the same A-a and B-b knob-hole interactions that allowed for initial dimer association. Protofibril growth continues, with branch points developing where multiple fibrin monomers interact laterally. The resulting fibrin polymer is a mesh-like network (Fig. 84.9).^{3,18}

Thrombin mediated release of fibrinopeptides from fibrinogen is neither calcium nor membrane dependent. However, fibrinogen contains several calcium binding sites. Calcium influences fibrin assembly, enhancing the rate and extent of lateral protofibril association. Consequently, calcium influences the structure of the mature fibrin clot.^{3,18} As the fibrin network forms, some thrombin is bound into the developing mesh via specific binding sites on the two molecules. Fibrin binding is important in activation of FXIII, and clot-bound thrombin also acts as a cofactor that accelerates fibrin assembly.^{3,17,18}

FIBRINOLYSIS

Fibrin clot formation prevents hemorrhage, but the clot must be removed eventually for restoration of blood flow. Clot dissolution is referred to as fibrinolysis. The fibrinolytic system is normally quiescent due to the relative abundance of fibrinolysis inhibitors, and the

requirement for fibrin as a cofactor for many of its processes. Although the primary function of fibrinolysis is removal of fibrin, many components of the system are involved in tissue repair processes, such as cell adhesion, migration, and proliferation.

Plasminogen

Plasminogen (PLG) is a single chain 92kDa glycoprotein synthesized primarily in the liver. It circulates in plasma with a long half-life of approximately 2 days. Its complex structure consists of five kringle domains and a protease domain. Several of the kringle domains bind aminocarboxylic acids (such as α_2 -aminocaproic acid and tranexamic acid) that are used as pharmacologic fibrinolysis inhibitors (see Chapter 90).

Plasminogen activators transform plasminogen to plasmin by cleavage at a single site, resulting in a two-chain molecule with an N-terminal glutamic acid residue (Glu-plasmin). *In vitro*, plasmin cleaves other plasmin molecules to remove part of the N-terminus, leaving an N-terminal lysine residue (Lys-plasmin). This additional cleavage probably does not occur *in vivo* due to rapid neutralization of free plasmin by inhibitors. The half-life of free plasmin is extremely short (0.1 seconds). Fibrin is a strong modulator of PLG activation. It enhances PLG activation due to binding and colocalization of PLG and its activators to the terminal lysine residues of fibrin monomers. Partially degraded fibrin exposes additional lysine sites, enhancing PLG activation through a positive feedback loop. Aminocarboxylic acids interfere with PLG binding to the lysine residues of fibrin. The fibrinolysis inhibitor, TAFI acts by removing lysine residues, thereby limiting available PLG binding sites.

The PLG molecule contains within its kringle domains the anti-proliferative protein angiostatin. Tumor mediated proteolysis or proteolysis by macrophages releases this fragment from plasmin. Angiostatin also inhibits endothelial cell and smooth muscle proliferation and migration, and induces apoptosis.⁵

Plasmin degrades polymerized fibrin to form heterogeneous fragments referred to collectively as fibrin degradation products (FDP). The D-dimer fragment is produced by degradation of the linked region between D domains of adjacent fibrin molecules. This linkage is created exclusively by FXIIIa; therefore the presence of D-dimers indicates that polymerized fibrin was subject to crosslinking.

Plasminogen Activators

Mammals produce two physiologic PLG activators, tPA and uPA, named for the source from which they were originally isolated (tissue and urine). Some bacteria and vampire bats also produce plasminogen activators.⁵

Tissue-type Plasminogen Activator

Tissue-type plasminogen activator is a 68kDa serine protease glycoprotein produced and secreted by endothelial cells in response to a variety of triggers,

including bradykinin, histamine, acetylcholine, α -adrenergic agents, and PAF. Very little tPA circulates in the bloodstream and most is bound to its major inhibitor, PAI-1. Free tPA and tPA-PAI-1 complexes are rapidly removed from circulation by binding to endothelial cell and hepatocyte receptors. The α_2 -MG-LRP interaction is a major clearance mechanism.

Endothelial cells secrete tPA as a single chain molecule (sctPA) that is *not* a zymogen; however, its enzymatic activity is weak in the absence of fibrin. Plasmin cleaves sctPA to form the two chain mature enzyme form (tPA). Fibrin binds to either single chain or two chain tPA. The protease activity of fibrin-tPA is strong and highly specific for cleavage of PLG to form plasmin.⁵

Urokinase Plasminogen Activator

Urokinase plasminogen activator is a 54 kDa serine protease glycoprotein synthesized by fibroblast-like cells, epithelial cells, monocytes, and endothelial cells. It is secreted as a single chain molecule (scuPA) with less than 1% protease activity of the two-chain mature enzyme, uPA. Plasmin, FXIIa, and kallikrein cleave scuPA to form uPA.⁵ Monocytes express an important cellular receptor for scuPA (uPAR). Colocalization of scuPA and uPAR on cell surfaces enhances PLG activation.

Unlike tPA, uPA can activate PLG in the absence of fibrin. It appears that tPA is the major PLG activator within the vasculature, whereas uPA is the major extravascular PLG activator. Consequently uPA's primary roles include degradation of extracellular matrix in the processes of cell migration, and healing.⁵

Fibrinolysis Inhibitors

Plasminogen Activator Inhibitor 1

Plasminogen activator inhibitor 1, a 52 kDa serpin, is the major inhibitor of PLG activators. The primary source of plasma PAI-1 is unknown; however, many cell types, including megakaryocytes, endothelial cells, and hepatocytes, synthesize PAI-1. It is produced as an unstable, active form found primarily in blood and tissues, and a more stable latent form present in platelets. Most of the circulating PAI-1 is complexed with vitronectin, which stabilizes PAI-1's active conformation.⁵²

The target enzymes of PAI-1 are sctPA, and the two chain forms of tPA and uPA. When bound to a PLG activator, PAI-1 loses its affinity for vitronectin, but gains affinity for cell-surface LRP. This shift results in rapid clearance of PAI-1 by internalization, mediated by LRP. Vitronectin-bound PAI-1 also inhibits thrombin, an activity enhanced by HSPG.⁵² In addition to its protease inhibitory activities, PAI-1 mediates vitronectin's adhesive properties and its role in tissue remodeling.^{5,52}

α_2 -Antiplasmin

α_2 -Antiplasmin is a single chain, 70 kDa serpin that is the primary plasmin inhibitor. It is synthesized in the liver, circulating with a long half-life of approximately 3 days.

It also binds to PLG, which interferes with PLG activation due to impaired PLG-fibrin interaction. Plasmin-bound fibrin is relatively resistant to α_2 -AP inhibition. Factor XIIIa cross-links some forms of α_2 -AP to fibrin, thereby making the fibrin more resistant to lysis.⁵

Thrombin Activatable Fibrinolysis Inhibitor

Thrombin activatable fibrinolysis inhibitor is a 60 kDa protein synthesized in the liver and released as a zymogen.⁶ Thrombin and plasmin activate TAFI by cleavage at a single site; however, activation is extremely slow. Thrombomodulin-bound thrombin, however, increases the TAFI activation rate more than 1000-fold. Activated TAFI inhibits fibrinolysis by removing the C-terminal lysines of polymerized fibrin. These residues are the primary binding site on fibrin for both PLG and its activators; therefore modification of fibrin by TAFIa makes the fibrin clot resistant to fibrinolysis. Complement cascade proteins and adhesive proteins are secondary targets of TAFIa.

No TAFIa inhibitors have been identified, rather TAFIa appears to be regulated via spontaneous loss of activity. This inherent lability is temperature dependent. At body temperature TAFIa has a half-life of about 10 minutes.

Miscellaneous Inhibitors

As a non-specific protease inhibitor, α_2 -MG acts to inhibit plasmin (especially in the absence of free α_2 -AP) and also binds to tPA and uPA. Protein C inhibitor and protease nexin 1 also inhibit tPA.

HEMOSTASIS AND INFLAMMATION

Recent discoveries demonstrate apparent "cross-talk" between the inflammatory and hemostatic systems. This communication plays important roles in the healing processes necessary for tissue repair, and in the inflammatory and innate immune response required when infection develops as a consequence of tissue injury. Cross-talk may be responsible for inappropriate activation of hemostasis associated with systemic inflammation (Chapter 88).

Effects of Hemostasis on Inflammation

Contact pathway activation produces two enzymes (kallikrein and FXIIa) that activate complement and induce neutrophil activation, and stimulate monocytes to down-regulate Fc receptors and release inflammatory cytokines. Additionally, cleavage products of HK have effects on cellular adhesion, cellular proliferation, and apoptosis.^{13,25,45}

The TF-FVIIa complex expressed on monocytes and endothelial cells activates cell surface PARs. This stimulation induces PAR-mediated signaling and increased expression of inflammatory cytokines (e.g. interleukins 1 and 6, tumor necrosis factor) and expression of chemotaxis proteins.^{13,25,45}

Thrombin is chemotactic for macrophages and neutrophils, stimulates selectin expression that promotes neutrophil adhesion to endothelial cells, and activates endothelial cells to produce PAF, a potent neutrophil agonist. Thrombin activation of platelets releases CD40 ligand which can induce TF formation and increase cytokine production. Thrombin also increases endothelial permeability.^{13,25,45}

The inflammatory response is attenuated by APC's enzymatic action on PAR-1, which down-regulates TF expression. Soluble EPCR, released by metalloproteinases from the endothelial cell surface, inhibits neutrophil adhesion. Down-regulation of TM expression increases endothelial intracellular signaling pathways responsible for production of proinflammatory cytokines, and limits activation of TAFI, adversely affecting clearance of some complement proteins.^{13,25,45}

Fibrin can directly stimulate production of proinflammatory cytokines by monocytes, and release of chemokines by endothelial cells and fibroblasts.^{13,25,45}

Effects of Inflammation on Hemostasis

Inflammatory mediators, especially components of the complement system, induce transmembrane phospholipid movement, resulting in expression of a procoagulant surface. The acute phase response results in a shift in the balance of protein synthesis. Proteins whose synthesis increases include fibrinogen, PLG, tPA, uPA, C1-INH, C4BP PS, vitronectin, PAI-1, and α_1 -PI. Proteins whose synthesis is decreased include FXII, AT, and PC.^{15,25}

Inflammatory cytokines down-regulate the expression of EPCR and TM on the endothelial cell surface. This results in less activation of APC, and consequently less inactivation of FVa and FVIIIa. Proinflammatory cytokines also induce expression of TF by cell-types that do not express this antigen under resting conditions. Proinflammatory cytokines also down-regulate expression of HSPG on the endothelial cell surface, which decreases the anticoagulant activity of AT.¹³ Neutrophil elastase cleaves a variety of coagulation and fibrinolysis proteins, including AT, PC, and TM.²⁵

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Acquired Coagulopathies

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Pathogenetic Classification

- Coagulation Factor Deficiencies of Acquired Coagulopathies
 - Synthetic failure
 - Activation defects
 - Factor consumption and dilutional coagulopathies
 - Coagulation Inhibitors
 - Fibrinolysis Defects
- ## Clinical Evaluation
- History
 - Physical Examination

- Quick Assessment and Point-of-Care Tests
- Coagulation Panel
- Specific Disorders
 - Liver Disease
 - Vitamin K Deficiency
 - Disseminated Intravascular Coagulation
 - Dilutional Coagulopathy
 - Coagulation Inhibitors
 - Factor VIII inhibitory antibodies
 - Antiphospholipid syndrome inhibitors
 - Heparin overdose

Acronyms and Abbreviations

ACT, activated clotting time; APLA, antiphospholipid-protein antibody; APS, antiphospholipid syndrome; aPTT, activated partial thromboplastin time; AT, antithrombin; DIC, disseminated intravascular coagulation; FDP, fibrin degradation product; KH₂, vitamin K hydroquinone; KO, vitamin K epoxide; PIVKA, proteins induced by vitamin K absence/antagonism; PT, prothrombin time; TCT, thrombin clotting time; TEG, thromboelastography; t-PA, tissue plasminogen activator; UFH, unfractionated heparin.

Clinical signs of hemorrhage are usually the result of a breach in vascular integrity that overwhelms an individual's normal hemostatic response. For these cases, diagnosis and management is directed at defining the site and cause of blood vessel injury. A subset of patients, however, have abnormal bleeding due to impaired hemostatic plug formation. Thrombocytopenia (see Chapters 78 and 79) and acquired coagulopathies are the most common hemostatic defects encountered in clinical practice. An index of suspicion and consistent use of screening tests early in the diagnostic work-up are key to recognition and effective management of acquired coagulopathies.

PATHOGENETIC CLASSIFICATION OF ACQUIRED COAGULOPATHIES

Acquired coagulopathies discussed in this chapter include disease processes that delay or prevent fibrin clot formation or increase the rate of fibrinolysis (Table 85.1).

Coagulation Factor Deficiencies

Coagulation factor activation generates a local burst of thrombin that transforms soluble plasma fibrinogen into an insoluble fibrin clot. Quantitative and/or functional deficiencies of coagulation factors influence the rate and amount of thrombin produced. (see Chapter 84). The common acquired factor deficiencies, unlike hereditary coagulopathies, are characterized by combined deficiencies of multiple clotting factors.

Synthetic Failure

The liver is the sole or primary source of all the serine protease coagulation factors and anticoagulants (i.e. factors II, VII, IX, X, XI, XII, proteins C and S) and other critical hemostatic proteins including factors V, XIII, antithrombin, and plasminogen. Liver disease is associated with complex abnormalities, with combined and variable deficiencies of procoagulant and anticoagulant proteins.^{1,25} Liver failure, however, typically manifests as a hemorrhagic diathesis. A lack of hepatic synthetic

TABLE 85.1 Pathogenesis of Acquired Coagulopathies

Mechanism	Disease Syndromes	Associated Factor Defects
Synthetic failure	Liver disease: severe failure and markedly reduced functional hepatic mass	Most coagulation factors low but relative retention of factor VIII activity Hypofibrinogenemia and dysfibrinogenemia Antithrombin and protein C low Protein C low
	Portosystemic shunting	
Vitamin K dependent activation defect	Cholestasis, intestinal malabsorption, Vitamin K antagonists, neonates	Factors II, VII, IX, and X low Protein C low
Excessive rate of consumption	Overt (hemorrhagic) disseminated intravascular coagulation	Complex pattern of increased and decreased factor activities, hypofibrinogenemia Antithrombin low
	Envenomation	Hypofibrinogenemia, variable deficiency of other factors
Coagulation inhibitors	Alloimmune and autoimmune factor inhibitors	Single factor deficiency (factor VIII deficiency most common)
	Phospholipid-protein antibodies (associated with thrombosis)	Coagulation factor activities normal
	Heparin overdose	Serine protease factors neutralized (especially factor X, factor II)
Hyperfibrinolysis	Disseminated intravascular coagulation, neoplasia, infectious agents, prostate surgery	Complex factor deficiencies High plasminogen activators Hypofibrinogenemia

capacity results in quantitative coagulation factor deficiencies, first detectable by decreased activities of factors with short plasma half-lives (e.g. factor VII, half-life of 6 hours). Plasma fibrinogen, as an acute phase reactant, is often normal or elevated in patients with inflammatory and cholestatic liver disease. Diseased hepatocytes, however, may produce a dysfunctional, “nonclottable” fibrinogen due to abnormal alpha chain conformation and high sialic acid content that impairs fibrin formation and polymerization. The development of hypofibrinogenemia in patients with liver disease is an indicator of severely compromised synthetic functional reserve, associated with poor prognosis.¹

Activation Defects

Vitamin K is essential for the post-translational processing of the prothrombin group of coagulation factors (factors II, VII, IX, X) and the anticoagulants, proteins C and S. All of these factors require the addition of gamma-carboxyglutamic acid residues in order to interact with calcium, bind to membrane surfaces, and form active enzyme complexes (see Chapter 84). Vitamin K participates in the carboxylation reaction through oxidation of vitamin K hydroquinone (KH_2) to vitamin K epoxide (KO). The epoxide form of vitamin K is continually recycled to replenish KH_2 by the enzyme vitamin K epoxide reductase (Fig. 85.1).

Coagulation factors synthesized in the absence of vitamin K circulate in plasma as inactive, descarboxy

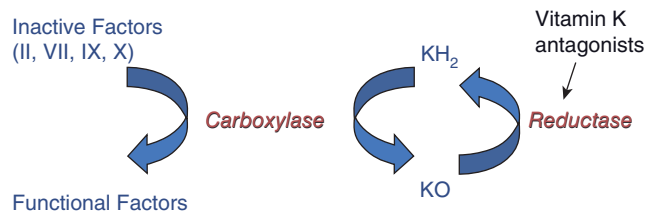


FIGURE 85.1 Vitamin K cycle. A vitamin K-dependent carboxylase simultaneously converts vitamin K quinol (KH_2) to vitamin K 2,3 epoxide (KO) as it transforms glutamate to functional gamma carboxyglutamate residues on factors II, VII, IX, and X. Vitamin K 2,3 epoxide is then reduced back to a quinol by vitamin K epoxide reductase. This enzyme is inhibited by coumarin-type anticoagulants.

precursors referred to as proteins induced by vitamin K absence or antagonism (PIVKA).

Factor Consumption and Dilutional Coagulopathies

Acute or chronic hemorrhage does not result in coagulation factor deficiency in animals with normal liver function. Factor consumption outpaces hepatic synthesis, however, in animals with overt disseminated intravascular coagulation (DIC). The resultant coagulation factor depletion causes clinical signs of hemorrhage (see Chapter 88). Systemic amyloidosis in people is associ-

ated with variable factor X deficiency, although the phenomenon has not been described in animals. Amyloid fibrils are believed to adsorb factor X and facilitate its rapid plasma clearance in some patients.⁷ The bleeding tendency of amyloidosis is multifactorial; due to amyloid tissue infiltration and deposition in the microvasculature, and potentially exacerbated by severe factor X deficiency (e.g. values below 25%). Many snake venoms contain proteases that activate clotting factors and/or directly cleave fibrinogen, resulting in systemic factor depletion and defibrination in a consumptive process.¹³

Dilutional hemostatic defects are common after aggressive fluid resuscitation or massive transfusion, commonly defined as replacement of more than one blood volume within a 24 hour time interval.²¹ Factor dilution develops secondary to fluid shifts from the interstitial and intracellular space into the intravascular space. These shifts are triggered by reduced intravascular hydrostatic pressure and are compounded by administration of large volumes of intravenous fluids. Activation of coagulation secondary to trauma-induced tissue factor expression also leads to factor consumption. Thrombocytopenia, acidosis, hypothermia and hypocalcemia (due to citrate-based anticoagulants in blood products) further contribute to hemostatic compromise.^{8,10}

Coagulation Inhibitors

The classification of coagulation inhibitors includes antibodies (usually IgG) directed against specific coagulation factors.¹⁹ The antibodies bind to and directly inhibit factor activity and/or speed the rate of factor clearance. Inhibitory alloantibodies develop in patients with hereditary factor deficiencies after transfusion exposure to the “foreign” antigen. Factor VIII inhibitory antibodies develop in approximately 20% of people with severe hemophilia A (factor VIII deficiency).^{19,20} Alloimmunization and development of factor VIII and IX inhibitors has also been described in dogs with hemophilia A and B, respectively.¹⁸ Autoantibodies directed against coagulation factors represent a loss of tolerance and can also develop in patients without hereditary factor defects, regardless of prior transfusion.⁹ Factor VIII is the most commonly implicated factor and the resultant coagulopathy is referred to as “acquired hemophilia.” The epitope specificity of factor VIII alloantibodies and autoantibodies generally overlap, with most binding to the protein’s light chain C domain. Recently, acquired factor V inhibitors have been recognized in people exposed perioperatively to topical bovine thrombin and fibrin glues.¹² These products contain trace amounts of bovine factor V, sufficient to induce cross-reactive antibody production, especially in multiply exposed patients.

The term “lupus anticoagulant” was formerly used to describe antibodies that prolong *in vitro* clotting times by blocking the interaction of coagulation factors with phospholipid reagents.¹⁵ The immunogenic epitopes are actually phospholipid binding proteins and the current terminology for these inhibitors is antiphospholipid-protein antibodies (APLAs).⁵ Para-

doxically, people with APLA develop signs of thrombosis, rather than hemorrhage. Although their prothrombotic action is not fully defined, APLAs are believed to displace natural anticoagulants on cell surfaces and induce exposure of procoagulant phospholipids in the vascular space.

Additional classes of coagulation inhibitors include pharmacologic anticoagulants such as hirudin that act by directly impairing factor activity and heparin compounds that act indirectly to enhance antithrombin’s ability to neutralize active factors (see Chapter 90). Finally, high plasma concentration of fibrin degradation products (FDPs) may act as coagulation inhibitors by interfering with normal fibrin polymerization.

Fibrinolysis Defects

Accelerated fibrinolysis causes hemorrhage by degrading fibrin clots before vascular repair is complete. Disease syndromes that induce increased plasminogen activators, decreased plasminogen inhibitors, or these factors combined, produce a hyperfibrinolytic state.²⁹ Hyperfibrinolysis, mediated by high tissue plasminogen activator (t-PA), develops in people infected with the agents causing Rocky Mountain spotted fever, African swine fever, and dengue fever, and secondary to extensive tissue trauma.⁸ Neoplasia is associated with hyperfibrinolysis due to specific elaboration of plasminogen activators by tumor cells (e.g. acute promyelocytic leukemia) or in the context of a DIC process (see Chapter 88). The prostate and uterus are rich in t-PA and urokinase plasminogen activators. Surgery on these organs risks hemorrhagic complications due to local hyperfibrinolysis and impaired intra- and post-operative hemostasis.

CLINICAL EVALUATION

The initial evaluation of animals with signs of hemorrhage should aim to differentiate blood loss caused by vessel injury or vascular disease from a systemic failure of normal hemostasis (i.e. bleeding diathesis). The location and nature of hemorrhage and prior history of abnormal hemorrhage or underlying disease conditions provide clues to guide subsequent laboratory evaluation. Some animals with acquired coagulopathies do not manifest overt hemorrhage at initial presentation. Detection of factor deficiency in these cases requires an index of suspicion and consistent inclusion of coagulation screening tests early in the diagnostic work-up.

History

A history of recurrent, episodic hemorrhage is suggestive of a hereditary hemostatic defect, rather than an acquired coagulopathy (see Chapters 81, 82, 86). Hemostasis questionnaires have been developed for medical studies with the goal of simplifying history gathering and generating bleeding “scores” for standard comparison among trials.²² A brief questionnaire with specific questions on hemostasis is also useful

early in the clinical work-up of animals suspected of having a bleeding disorder. Pertinent questions include: Has this animal ever had spontaneous bleeding from the nose, mouth, urinary tract, or noticeable bleeding when teething? Has this animal ever had a surgical procedure, dentistry, or traumatic injury, and were there any bleeding complications? Are you aware of any bleeding problems in relatives or in this breed? Is this animal receiving any medication or dietary supplements? Does this animal have any ongoing medical problems? Additional questions regarding housing conditions and potential access to drugs and toxins are useful; however, a negative exposure history does not preclude toxicity.

Physical Examination

The physical examination should define focal or multifocal sites of hemorrhage and detect evidence of gross and subtle hemorrhage. Funduscopic examination and careful inspection of mucous membranes and non-haired skin may reveal petechiae and ecchymoses, suggestive of platelet or von Willebrand factor defects or vasculopathies. Large vessel (arterial/venous) injury typically causes obvious blood loss from localized sites of vessel injury. Animals with severe coagulopathies may develop spontaneous hemorrhage into any body cavity or potential space, producing effusion, mass effect, or hematoma formation. Simultaneous hemorrhage from several sites, including prolonged hemorrhage from venipuncture or catheter sites is evidence of severe coagulopathy. The possibility of an acquired coagulopathy should be included in the differential for any patient with unexplained hemorrhage, effusion or mass lesion, or disease conditions associated with factor deficiencies (see Table 85.1). Routine coagulation screening of these cases will minimize delays in instituting appropriate management.

Quick Assessment and Point-of-Care Tests

The initial assessment of any patient suspected of having a hemostatic defect should include platelet count or platelet estimate (see Chapter 77) and a point-of-care coagulation test. The activated clotting time (ACT) is the traditional, and simplest coagulation screening test (see Chapter 138). The ACT detects moderate to severe deficiencies of fibrinogen and all coagulation factors, with the exception of factor VII. The ACT is prolonged, therefore, in patients with active hemorrhage due to most acquired coagulopathies, including liver failure, anticoagulant rodenticide intoxication, DIC, and heparin overdose (see Table 85.1). More advanced instruments are now available to perform additional point-of-care clotting time tests using whole blood samples. A clinical study of coagulopathies in dogs found similar diagnostic utility comparing results of laboratory and point-of-care coagulation panels.²⁶ Recently, viscoelastic monitors have been developed with the goal of providing a global assessment of hemostasis that incorporates the contribution of cellular elements and procoagulant and anticoagulant plasma

proteins.⁶ The thromboelastography (TEG) analyzer is the viscoelastic instrument used most widely to study coagulopathies in dogs.^{18,28} This instrument provides a visual tracing and numeric parameters reflecting the rate and strength of fibrin formation. Clinically significant coagulopathies are expected to cause prolonged initiation times and diminished tracing amplitude. Evidence of hypocoagulability, in either simple or sophisticated point-of-care tests, requires follow-up with specific coagulation panel assays (and possibly more detailed factor and anticoagulant assays) for definitive diagnosis of acquired coagulopathies.⁶

Coagulation Panel

The basic screening tests to detect coagulopathies include activated partial thromboplastin time (aPTT), prothrombin time (PT), thrombin clotting time (TCT) and fibrinogen concentration (see Chapter 138). The assays are functional tests, configured with specific reagents that sequentially activate distinct series of coagulation factors (i.e. aPTT and PT) or with a thrombin-containing reagent that directly transforms plasma fibrinogen to fibrin (i.e. TCT and fibrinogen). Factor deficiencies are detected by prolongation of clotting times. The pattern of abnormalities in screening test results depends on which factor(s) the patient lacks (see Fig. 85.2).

Coagulation panel results, combined with clinical presentation, are often sufficient for management of acquired coagulopathies. Complex cases may require specific analyses of individual coagulation factors, anticoagulant proteins, drugs, or measures of fibrinolysis, to better define pathogenesis or facilitate case management. Collection of pre-treatment samples is critical for timely and accurate diagnosis of coagulopathies, and clinicians must bear in mind that the validity of all hemostasis testing depends on appropriate sampling techniques (see Chapter 138).

SPECIFIC DISORDERS

Liver Disease

Animals with hepatobiliary disease demonstrate variable signs of coagulopathy, ranging from spontaneous bleeding to subclinical prolongation of *in vitro* clotting times.^{2,14,24} Liver disease causes major alterations in many hemostatic mechanisms, including platelet and endothelial cell reactivity, procoagulant and anticoagulant protein production and function, dysregulation of fibrinolysis, and complications arising from portal hypertension and splenomegaly. These changes often act as opposing procoagulant and anticoagulant forces, resulting in variable net bleeding risk. Clinically severe hemorrhage is generally seen in animals with fulminant or end-stage hepatic failure, or those with concomitant DIC. Other forms of liver disease may cause relatively milder bleeding tendencies, albeit sufficient to impair surgical hemostasis for animals undergoing biopsy or hepatobiliary surgery.

Diagnostic Algorithm for Acquired Coagulopathies

COAGULATION SCREENING TESTS: APTT, PT, TCT

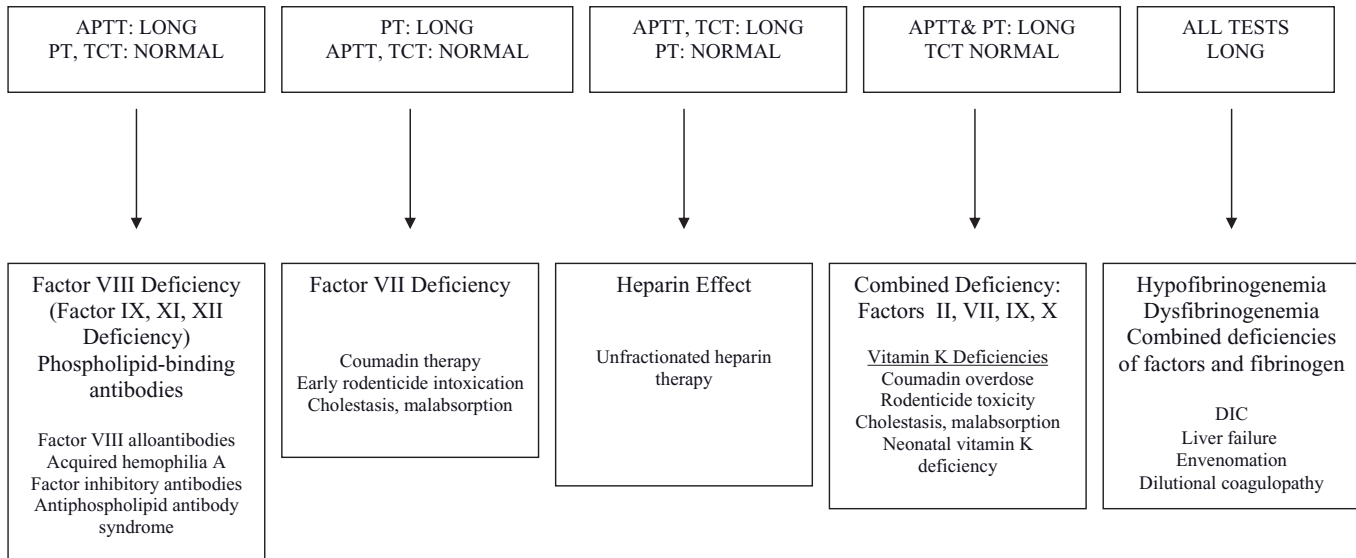


FIGURE 85.2 Coagulation screening test algorithm for acquired coagulopathies.

Marked prolongation of coagulation screening tests (aPTT, PT, TCT) and hypofibrinogenemia indicate high risk of spontaneous and post-surgical hemorrhage, and are overall poor prognostic signs. Mild prolongation or the absence of abnormal clotting times, however, does not rule out the risk of procedure-related bleeding. Newer hemostasis tests, such as endogenous thrombin potential and thromboelastography are designed to assess net procoagulant and anticoagulant forces. Their utility for predicting bleeding risk in patients with liver disease is currently under clinical investigation.⁶ A preliminary study has shown that measurement of protein C activity may be useful as a biomarker of liver function and hepatoportal blood flow in dogs.²⁴ The finding of low protein C activity helps differentiate porto-systemic vascular anomalies from microvascular dysplasia, and is a useful predictor of clinical outcome when combined with traditional biochemical assays.

Management strategies for coagulopathy of liver disease include prophylactic and “rescue” therapies. Prophylactic administration of vitamin K₁ is often included with specific and supportive medical therapy for liver disease. Pre-operative vitamin K₁ administration is indicated for animals with cholestatic disorders and/or prolonged aPTT and PT (see Chapter 90). Current trends in human medicine favor more restrictive use of prophylactic transfusion therapy.¹ Fresh

frozen plasma is transfused to patients with liver failure and signs of hemorrhage to supply active procoagulant and anticoagulant factors (see Chapters 90 and 96). The need for pre-operative transfusion is generally assessed on an individual patient basis in human and veterinary medicine. People undergoing liver transplantation invariably require transfusion and algorithms based on thromboelastographic parameters have been developed to guide the use of blood components in this patient population.⁶

Vitamin K Deficiency

Vitamin K deficiency is a common cause of coagulopathy, resulting from inadequate intestinal absorption or impaired intrahepatic recycling.^{2,4,14,16} Biliary obstruction, intrahepatic cholestasis, chronic oral antibiotic administration, and infiltrative bowel disease may all reduce vitamin K absorption. Hemorrhagic disease of the newborn refers to vitamin K deficiency in infants within the first few days to weeks of life. Hemorrhage is attributed to inadequate placental transfer of vitamin K and inadequate hepatic recycling.¹¹ Coumadin (warfarin) and second-generation anticoagulant rodenticides (e.g. brodifacoum) act by irreversibly blocking the activity of epoxide reductase, the critical hepatic vitamin K recycling enzyme (see Fig. 85.1). Severe coagulopathies due to anticoagulant rodenticide toxicity are com-

monly encountered in small animal practice, have been reported in equine practice, and are increasingly recognized in wild birds (see Chapter 91). Factor deficiency and resultant signs of a bleeding diathesis develop over a period of several days post-ingestion of anticoagulant poisons as vitamin K stores become depleted. Signs of hemorrhage may be obvious as external blood loss, or may be nonspecific as pleural or abdominal effusion or mass lesions anywhere in the body due to hematoma formation. Treatment delays often lead to death from hemorrhagic shock, or respiratory or central nervous system compromise.

Prolongation of both the aPTT and PT screening tests, with normal TCT, are the hallmarks of hemorrhage caused by severe vitamin K deficiency (see Fig. 85.2). Specific factor analyses reveal that severe deficiencies of factors II, VII, IX, and X but normal fibrinogen cause this characteristic coagulation profile. Therapeutic dosages of the anticoagulant Coumadin cause only mild deficiencies of the vitamin K-dependent factors that should not cause prolongation of the aPTT. Coumadin monitoring to ensure adequate, but not excessive dosage, is based on PT analyses (see Chapter 90).

Management of vitamin K deficiency requires replacement therapy with vitamin K₁ (phytonadione), rather than vitamin K₃ (menadione). Vitamin K therapy is often given empirically pending results of pre-treatment coagulation assays, because synthesis of sufficient active factors to correct the coagulopathy requires at least 12 hours from the start time of initiating vitamin K supplementation. Transfusion is also indicated for patients with severe signs at presentation (see Chapter 96).

Disseminated Intravascular Coagulation

Microvascular thrombosis is the characteristic feature of DIC; however, signs of a severe bleeding diathesis dominate some case presentations. Dogs with DIC secondary to neoplasia (e.g. hemangiosarcoma) often develop consumptive coagulopathies, with coagulation factor and fibrinogen depletion, and concomitant increase in FDP and D-dimer (see Chapter 88). Hemorrhagic, overt DIC is often transfusion resistant, requiring high volume plasma replacement to sustain hemostasis (see Chapter 96).

Dilutional Coagulopathy

The terms dilutional coagulopathy, or “traumatic coagulopathy” describe a hemostatic defect that develops after high volume fluid resuscitation. Traumatic coagulopathy occurs in up to 1 of 4 human trauma patients and is considered a negative prognostic indicator. Concomitant acidemia, hyperfibrinolysis, and hypothermia further exacerbate coagulopathy in these patients. The effect of crystalloids versus colloids in promoting a dilutional coagulopathy has been extensively studied, with colloids demonstrating a greater dilutional effect than crystalloids and a negative effect on clot stability.⁸ Clinical studies in people also reveal

that fibrinogen deficiency generally develops early in the course of hemodilution and signs of inadequate hemostasis manifest at fibrinogen concentration below 50–100 mg/dL (0.5–1.0 g/L).²¹ Although traumatic coagulopathies are not as well-characterized clinically in animals, a retrospective study of massive transfusion in dogs noted prolonged aPTT and PT in half the cases.¹⁰ Correction of hemostatic abnormalities is associated with improved survival in people, and factor replacement in the form of fresh frozen plasma is typically administered after recognition of coagulopathy.⁸

Coagulation Inhibitors

Factor VIII Inhibitory Antibodies

Antibodies that inhibit factor VIII are a well-recognized complication of factor replacement therapy for people with hemophilia A. This alloantibody formation is commonly, but not exclusively, found in patients with severe factor VIII deficiency (i.e. $\leq 1\%$ factor VIII).¹⁹ Consequently, mutation types that abolish protein synthesis appear to increase the risk of inhibitor formation. The development of coagulopathy due to factor VIII inhibitory antibodies also occurs as an autoimmune phenomenon.⁹ Underlying conditions associated with acquired factor VIII inhibitor development include systemic lupus erythematosus, rheumatoid arthritis, drug hypersensitivity, inflammatory bowel disease, malignancy, and the post-partum state. Factor VIII inhibitors (alloantibodies and autoantibodies) often cause severe coagulopathies characterized by spontaneous hemorrhage refractory to standard replacement therapy.

Factor VIII inhibitors are detected by specific prolongation of the aPTT. This prolongation is not corrected by mixing patient plasma with normal plasma, in contrast to congenital factor VIII deficiency (in the absence of factor VIII inhibitors). The Bethesda unit assay (BU) is used to quantify the titer of inhibitory antibodies. Values below 5 BU/mL are considered low titers.^{19,20} Active bleeding in patients with low BU titers often responds to transfusion of high dose factor VIII replacement therapy. In contrast, patients with high titer inhibitors often require “by-pass” therapy using recombinant activated human factor VII (see Chapter 96). Long-term management of high titer factor VIII inhibitors consists of specific therapy of underlying disorders (for autoantibody patients) and immune-modulating or immunosuppressive therapy.²⁰

Antiphospholipid Syndrome Inhibitors

The antiphospholipid syndrome (APS) is recognized as a thrombotic risk factor in people. The syndrome typically develops in patients with autoimmune disorders and is characterized by the production of APLAs that prolong *in vitro* clotting time.¹⁵ Long aPTT that fails to correct in a mixing study is compatible with the presence of APLA, and more specialized clotting time tests (e.g. dilute Russell’s viper venom time) and quantitative immunoassays to detect antibodies directed against

lipid-binding proteins are used as confirmatory assays.⁵ Inhibitory antibodies were proposed as the cause of long clotting times in a case report of canine immune-mediated hemolytic anemia (IMHA) and thrombosis; however, the prevalence and clinical relevance of APLA and APS in animals is not well-defined.²³

Heparin Overdose

Unfractionated heparin (UFH) is widely used in veterinary medicine; however, few clinical studies have been performed to define effective dosage regimens. The pharmacokinetic profile of UFH is complex due to its extensive protein and cell binding and dose-dependent half-life (see Chapter 90).^{3,17,27} Since hemorrhage is the major risk of high dose UFH therapy, individual patient monitoring is required to prevent excessive anticoagulant effect. The aPTT and TCT coagulation screening tests are both sensitive to UFH, whereas the PT is generally unaffected by therapeutic UFH levels. Adjustment of UFH dose to prolong aPTT to 1.5–2 times pretreatment values is the traditional means for guiding UFH therapy in people, and this strategy is applicable to prevent overdosage in animals. Clinical signs of hemorrhage or marked prolongation of aPTT (i.e. >2.5 times baseline) are indications for discontinuing UFH therapy. Due to its short (2–3 hour) plasma half-life, hemorrhage is usually transient. People with major hemorrhage due to UFH overdosage are infused with protamine sulfate, a basic polypeptide that binds tightly to UFH and immediately neutralizes its anticoagulant activity.²⁷ Protamine administration causes systemic hypotension in dogs; therefore transfusion therapy is used to control life-threatening hemorrhage caused by excessive UFH anticoagulant effect (see Chapter 96).

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ON-LINE RESOURCES

1. **Comparative Coagulation Laboratory.** Animal coagulation, anticoagulant, and fibrinolysis assays. <http://www.diaglab.vet.cornell.edu/coag/>
2. **Massachusetts General Hospital.** Overview of assays to aid in diagnosis of coagulopathies. <http://www2.massgeneral.org/pathology/coagbook/handbook.htm>

Hereditary Coagulopathies

MARJORY B. BROOKS

Hereditary Coagulation Factor Deficiencies

X-Linked Factor Deficiencies: Hemophilia A and B

Clinical Diagnosis of Hemophilia (see Chapter 138)

Treatment of Hemophilia (see Chapters 90, 96)

Genetics

Autosomal Factor Deficiencies

Fibrinogen Deficiency

Prothrombin Deficiency

Factor VII Deficiency

Factor X Deficiency

Factor XI Deficiency

Factor XII and Contact Factor Deficiencies

Combined Deficiencies of Vitamin K-dependent Factors

Unrecognized Factor Deficiencies

Acronyms and Abbreviations

ACT, activated clotting time; aPTT, activated partial thromboplastin time; coagulation factors: FII, factor II; FVII, factor VII; FVIII, factor VIII; FIX, factor IX; FX, factor X; FXI, factor XI; FXII, factor XII; FXIII, factor XIII; DIC, disseminated intravascular coagulation; HMWK, high molecular weight kininogen; PIVKA, proteins induced by vitamin K absence/antagonism; OMIA, on-line Mendelian inheritance in animals; OMIM, on-line Mendelian inheritance in man; PK, prekallikrein; PT, prothrombin time; RVVT, Russell's viper venom time; TCT, thrombin clotting time; VKOR, vitamin K epoxide reductase.

HEREDITARY COAGULATION FACTOR DEFICIENCIES

Hereditary coagulopathies arise from mutations within genes required for synthesis or processing of active coagulation factors. The bleeding tendency associated with each trait depends on the physiologic role of the deficient factors in fibrin clot formation and the degree of factor deficiency. Hemophilia A is the most common hereditary coagulopathy in human beings and animals, due in part to a high de novo mutation rate in the coagulation FVIII gene (*F8*).¹¹ In addition, new factor deficiencies continually arise within breed populations with variable frequencies that change over time. The general breeding strategies to limit propagation of hereditary factor defects include exclusion of individuals expressing a bleeding tendency, avoidance of repeat matings that produce affected individuals, and familial screening (especially dams, sires, siblings) of affected patients to identify others with quantitative or functional factor deficiencies. In addition to the review of defects presented in this chapter (Table 86.1), public access databases, such as Online Mendelian Inheritance in Man and Animals (OMIM, OMIA) hosted by the

NIH, provide information on hereditary factor deficiencies in animals.

X-LINKED FACTOR DEFICIENCIES: HEMOPHILIA A AND B

Hemophilia A (OMIM 306700) and hemophilia B (OMIM 306900) are distinct X-linked bleeding disorders caused by functional or quantitative deficiencies of FVIII and FIX, respectively.¹¹ Although both genes map to the long arm of the X chromosome, they are inherited independently. Factor IX (a serine protease enzyme) and FVIII (its coenzyme) play critical roles in the amplification phase of coagulation by participating in formation of the "tenase" enzyme complex (see Chapter 84).

Clinical Diagnosis of Hemophilia

Hemophilia A and B are diagnosed primarily in males because of the sex-linked, recessive inheritance pattern of these traits. Carrier (heterozygous) females do not express a bleeding tendency. Signs of hemophilia include lameness due to hemarthrosis, intramuscular

TABLE 86.1 Hereditary Factor Deficiencies

Factor Deficiency	Screening Test Results		Species (Breeds)
	Abnormal	Normal	
Fibrinogen	TCT, ^a fibrinogen ^a All tests ^b	*aPTT, *PT	Dogs (Bichon Frise, Borzoi, collie), cats (DSH), goat (Saanen), sheep (Leicester)
Factor II (prothrombin) Factor VII (proconvertin)	aPTT, PT PT	TCT, fibrinogen aPTT, TCT, fibrinogen	Boxer Dogs (Alaskan Klee Kai, Beagle, Deerhound, Malamute, Schnauzer), cats (DSH)
Factor VIII (hemophilia A)	aPTT	PT, TCT, fibrinogen	Dogs (any breed, mixed breeds, German shepherd, Golden retriever), cats (any breed, DSH), horse, sheep
Factor IX (hemophilia B)	aPTT	PT, TCT, fibrinogen	Dogs (any breed, mixed breeds), cats (any breed, DSH), horse
Factor X (Stuart Prower factor)	aPTT, PT	TCT, fibrinogen	Dogs (Cocker spaniel, Jack Russell terrier), cats (DSH)
Factor XI (PTA deficiency, hemophilia C)	aPTT	PT, TCT, fibrinogen	Dogs (English springer spaniel, Kerry blue terrier), cats (DSH)
Factor XII (Hageman factor)	aPTT	PT, TCT, fibrinogen	Dogs (Miniature poodle, Shar Pei), cats (DSH, DLH, Siamese, Himalayan)

^aMild fibrinogen deficiency.

^bSevere fibrinogen deficiency.



FIGURE 86.1 Clinical signs of hemophilia. Intramuscular hematoma formation in the lateral thigh muscles of a hemophilic German shepherd. Note distal limb swelling due to edema, venous obstruction, and extravasated blood.

and subcutaneous hematoma formation, and prolonged hemorrhage from minor wounds and gingiva at tooth eruption sites (Fig. 86.1). Severe hemophilia typically manifests within the first few months of life. Severely affected patients are at risk for spontaneous and fatal hemorrhage, whereas milder hemophilia may become apparent only after surgery or trauma.

Hemophilia A is the more common form in all species, with an incidence of 1–2 cases per 10,000 male births (in human populations). Unlike many hereditary diseases in animals, hemophilia is not restricted to a single breed or inbred line. Cases of hemophilia A and B have been identified in mixed breed dogs and more than 50 different purebred lines.² Both traits have been reported in domestic and purebred cats^{9,14} and horses,⁶ and hemophilia A has been characterized in sheep.¹

The laboratory diagnosis of hemophilia is based on results of coagulation assays. Both forms of hemophilia cause specific prolongation of intrinsic pathway screening tests (ACT and aPTT), but do not affect tests of the extrinsic/common pathways or fibrinogen (see Table 86.1). Definitive diagnosis and differentiation of hemophilia A from hemophilia B requires specific measurements of FVIII and FIX coagulant activities (FVIII:C, FIX:C). Clinical severity relates to residual factor activity. Severe hemophilia is characterized by FVIII:C or FIX:C < 2% of normal, whereas factor activities of 2–20% are associated with moderate to mild hemophilia. Factor assay values are derived from comparisons of patient plasma with the activity of plasma standards. Reaction kinetics vary among species; therefore animal factor activity assays are best performed using same-species plasma standards (see Chapter 138).

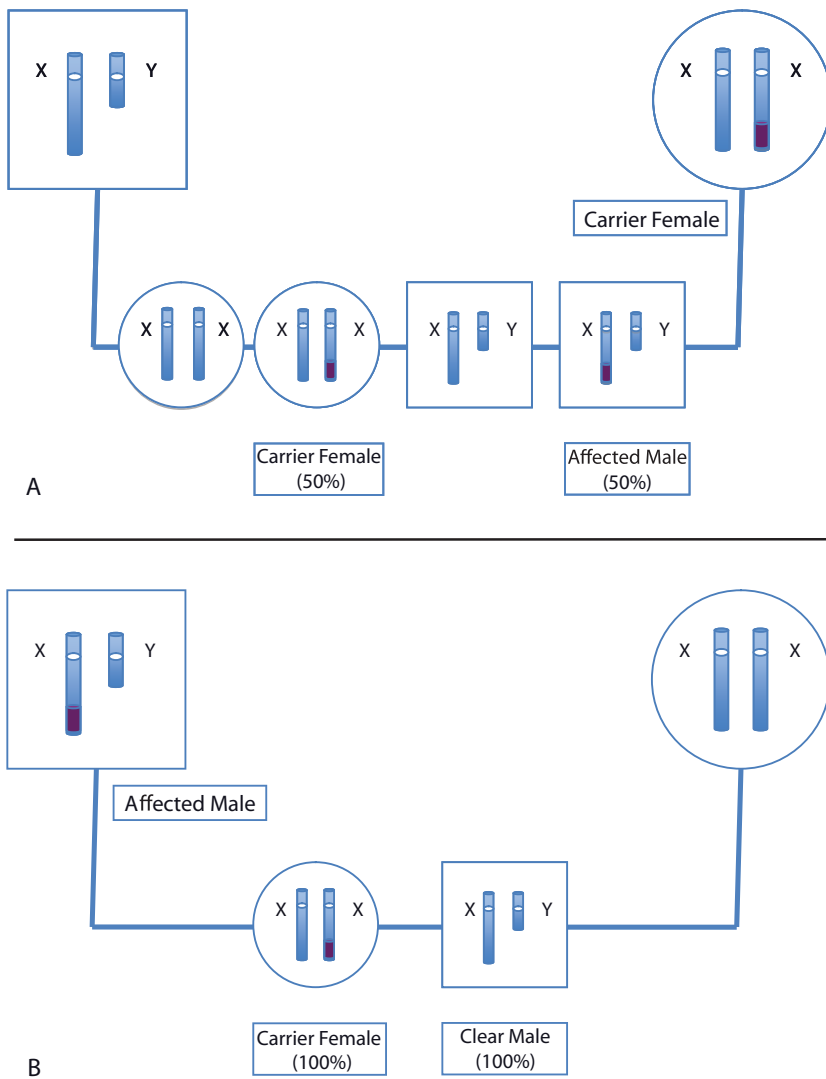


FIGURE 86.2 Inheritance of hemophilia A and B. (A) Propagation of hemophilia by a carrier female. If a carrier female is bred, on average one-half of her daughters and one-half of her sons will inherit her mutant gene (depicted as a purple bar on the X chromosome). (B) Propagation of hemophilia by an affected male. Males with mild forms of hemophilia may survive to reproduce. If bred, they transmit a mutation to all their daughters and none of their sons.

Treatment of Hemophilia

Transfusion is the primary means for controlling or preventing hemorrhage in animals affected with hemophilia A or B (see Chapters 88 and 96). Fresh frozen plasma supplies both FVIII and FIX, whereas cryoprecipitate is a more specific product for replacement of FVIII, and cryosupernatant supplies FIX. The transfusion interval for hemophiliacs varies depending on residual factor activity, and clinical features such as patient size, activity, and conditions that further impair hemostasis. Maintenance of patients with severe hemophilia (<2% FVIII:C, FIX:C) is difficult due to recurrent hemorrhage. Many severely affected animals are euthanized due to poor quality of life, or experience fatal bleeds before 1 year of age.

Genetics

New cases of hemophilia arise from frequent de novo mutation events in the FVIII and FIX genes. Once

present, hemophilia mutations are typically transmitted by asymptomatic carrier dams; however, males with mild forms of hemophilia may survive to adulthood and widely propagate the trait if they are bred (Fig. 86.2). Males are accurately diagnosed as affected or clear of hemophilia based solely on FVIII and FIX assays. Obligate carrier females are then identified by pedigree review as the dams and daughters of the affected males. The status of suspect carrier females is difficult to classify with factor assays, however, because values overlap for carrier and clear females at the low end of the normal range. Molecular genetic analyses of hemophilia indicate that unique mutations within the FVIII and FIX genes are likely causative for hemophilia in different breeds and families.² In support of this prediction, distinct FIX mutations have been identified in different breed-variants of canine and feline hemophilia B.^{3,14} The discovery of hemophilia A causative mutations is more challenging because of the large size and complexity of the FVIII gene. Direct mutation detection is the most accurate means of hemophilia carrier detec-

tion, providing the underlying mutation is known. An alternate, indirect strategy utilizing DNA markers linked to the FVIII gene has proven feasible for carrier detection of hemophilia A in sheep and dogs.^{1,5}

AUTOSOMAL FACTOR DEFICIENCIES

All of the remaining factor deficiencies identified in animals (fibrinogen, FII, VII, X, XI, XII, and contact factor defects) are less common than hemophilia, and are typically restricted to a few breeds or families (Table 86.1). These autosomal defects are primarily recessive traits. In this form of inheritance, males and females express and transmit the traits with equal frequencies, affected individuals are homozygous for the disease-causing mutation, and heterozygous carriers do not express a bleeding tendency (Fig. 86.3).

Fibrinogen Deficiency

Fibrinogen is the plasma precursor of fibrin and also supports intra-platelet bridging in the process of aggregation. Fibrinogen consists of three polypeptide chains (A- α , B- β , and γ), coded for by three closely linked genes. Mutations in any of these genes can impair fibrinogen function or secretion.

Many distinct hereditary fibrinogen defects have been identified in people, broadly classified as quantitative deficiencies (i.e. afibrinogenemia and hypofibrinogenemia) and functional defects (dysfibrinogenemia and hypodysfibrinogenemia) (OMIM 202400). Afibrinogenemia is rare and causes a severe bleeding diathesis. Signs of dysfibrinogenemias are variable, ranging from severe hemorrhage to thrombosis. Hereditary fibrinogen defects have been identified in several breeds of dogs, and in cats, goats, and sheep (Table 86.1).^{10,26} Reported manifestations include severe, spontaneous hemorrhage and hemarthrosis. Milder forms are characterized primarily by post-traumatic and post-surgical hemorrhage.

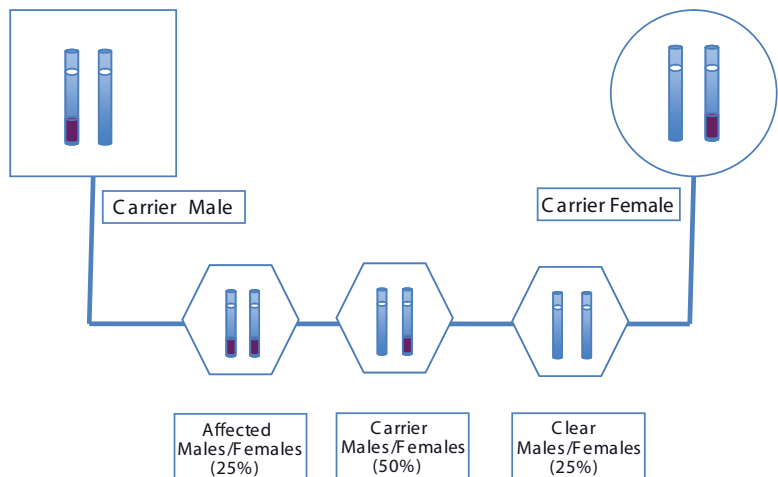
Fibrinogen deficiency affects formation of a fibrin clot endpoint in all clotting time tests (Table 86.1). The TCT is the most sensitive of these screening tests to detect mild fibrinogen deficiencies. A combination of functional (clottable) fibrinogen assays and quantitative methods, such as enzyme-linked immunosorbent assay (ELISA), are required for definitive diagnosis of hypo/dysfibrinogenemias and afibrinogenemia (see Chapter 138).

Hereditary fibrinogen defects are much less common than acquired hypofibrinogenemia, which often develops secondary to hepatic synthetic failure or consumption in a DIC process (See Chapter 85). Unlike acquired disorders, hereditary fibrinogen deficiencies are rarely associated with concomitant thrombocytopenia, or deficiencies of other coagulation factors. The absence of clinical and laboratory signs of hepatic failure or DIC further aid in differentiating hereditary from acquired fibrinogen defects.

Cryoprecipitate is the treatment of choice for fibrinogen defects as it supplies high concentration fibrinogen in low volume transfusion. Fresh frozen plasma is an alternative product if cryoprecipitate is unavailable (see Chapter 96). Transfusion is indicated for patients with active hemorrhage, or for preoperative prophylaxis. The human dosage recommendation is transfusion to a target of 50–100 mg/dL fibrinogen.²¹ The half-life of fibrinogen of 2–4 days is relatively long compared with most factors; thus fewer transfusions at longer intervals are often sufficient to support hemostasis for patients with fibrinogen defects.

Fibrinogen defects in people are mutationally heterogeneous and display autosomal dominant and recessive inheritance patterns. Afibrinogenemia is usually a recessive trait caused by mutations in the fibrinogen A- α chain. Hypo/dysfibrinogenemias have been described in patients with mutations in all three fibrinogen genes. Pending pedigree studies and molecular analyses, it is likely that animals will display the same variety of disease-gene, mutation location, and mutation-type found in human patients.

FIGURE 86.3 Inheritance of autosomal recessive factor deficiencies. Mutant genes (depicted as purple bars on an autosome) are transmitted from dam and sire to male and female offspring with equal frequencies. On average, one-quarter of the offspring will be homozygous (affected with the defect), one-half of the offspring will be asymptomatic heterozygous carriers, and one-quarter of offspring will inherit no mutant gene and therefore be clear of the trait.



Prothrombin Deficiency

Hereditary FII (prothrombin) deficiency is uncommon, with fewer than 100 cases reported in human beings and isolated cases in a few breeds of dog (OMIM 176930).¹⁰ Prothrombin is the zymogen of thrombin, the serine protease factor that transforms fibrinogen to fibrin. Thrombin also plays a central role in platelet activation, inflammation, and wound healing. Knock-out mice lacking the prothrombin gene are nonviable and all naturally occurring human (and animal) cases have some residual thrombin activity.

Clinical signs of hemarthrosis and post-traumatic hemorrhage are generally seen in patients with residual thrombin activities below 10% of normal. Prothrombin deficiency causes prolongation of the aPTT and PT (and PIVKA) screening tests, but does not affect the TCT or fibrinogen assay (Table 86.1). Differential diagnoses for this pattern of screening test abnormalities include deficiency of a single common pathway factor (FII, FV, FX) and combined deficiency of all vitamin K-dependent factors (FII, FVII, FIX, FX). The finding of specific deficiency of FII coagulant activity (FII:C), with normal activity of the other factors, provides definitive diagnosis of prothrombin deficiency (see Chapter 138).

Fresh frozen plasma and plasma cryosupernatant supply FII. The half-life of FII is approximately 3 days, and transfusion to attain target FII:C of 20–40% is the recommended guideline to maintain hemostasis in human patients.²¹

The expression of FII deficiency in people is autosomal recessive; affected patients are homozygous with residual FII:C ranging from 2% to 20%. Heterozygotes have FII:C of approximately 50% and are clinically asymptomatic. Although new mutations could arise, FII deficiency is uncommon and rarely recognized in any breed at this time.

Factor VII Deficiency

Trace amounts of activated FVII (FVIIa) bind to membrane-bound tissue factor at sites of vascular injury. This FVIIa/tissue factor complex plays a central role in the initiation phase of coagulation. Hereditary FVII deficiency is often associated with a clinically mild or inapparent bleeding diathesis, suggesting that sufficient residual FVII remains for active complex assembly. The trait has been identified in people and several different breeds of dog (OMIM 227500).⁷

Canine FVII deficiency is best characterized in Beagles.⁷ Factor VII deficient Beagles rarely experience abnormal bleeding, even after surgery or trauma. In contrast, FVII deficiency in Malamutes is typically associated with more severe signs including hematoma formation, prolonged post-traumatic bleeding, and fatal hemorrhage.² Clinical severity may vary among individuals within a single breed. In a recent report of FVII deficient Alaskan Klee Kai, the index case experienced prolonged post-traumatic hemorrhage whereas other related dogs had undergone major surgery with no complications.¹⁶

Factor VII deficiency causes an isolated prolongation of the PT (and PIVKA) screening tests, with normal aPTT, TCT, and fibrinogen (Table 86.1). Hereditary FVII deficiency is differentiated from vitamin K-dependent coagulopathies as a single factor, rather than a combined multi-factor defect. In most canine and human kindreds, FVII deficient patients have some residual FVII activity with FVII coagulant activity (FVII:C) generally ranging from 1% to 10% of normal. More detailed laboratory characterization of residual FVII includes measurement of FVII antigen and levels of circulating FVIIa (see Chapter 138).

Most FVII deficient patients are transfusion-independent; however, those with severe forms and signs of hemorrhage can be transfused with fresh frozen plasma or cryosupernatant to supply FVII. Factor VII has a short plasma half-life of approximately 3–4 hours; therefore transfusion intervals of 8–12 hours may be needed to sustain hemostasis after initial stabilization (see Chapter 96).

Factor VII deficiency is a recessive trait. A mis-sense mutation in exon 5 of the FVII gene was first identified in FVII deficient Beagles, and subsequently found to cause FVII deficiency in Alaskan Klee Kai and Deerhounds.^{7,16} Heterozygous carriers of the mutation generally have FVII:C values intermediate between affected and clear dogs. Direct mutation detection is preferable for carrier detection because factor activities overlap between carrier and clear individuals. Mutations causative for FVII deficiency in other breeds or species have not yet been identified.

Factor X Deficiency

Factor X and its cofactor, FV, play a central role in generating thrombin. The factors assemble on cell membrane surfaces to form the prothrombinase complex which rapidly converts prothrombin to the active enzyme, thrombin (see Chapter 84).

An absolute lack of FX is an apparently lethal trait; however, partial FX deficiency has been identified in human beings, dogs, and a DSH cat (Table 86.1; OMIM 227600).^{8,10,13} The defect is a relatively severe bleeding diathesis in these species, with signs of spontaneous epistaxis, hematoma formation, and severe hemorrhage post-trauma or surgery.

Factor X deficiency causes prolongation of the aPTT, PT, PIVKA, and RVVT screening tests; however, TCT and fibrinogen are normal (see Chapter 138). Clotting time in the RVVT is initiated by direct activation of FX, independent of the intrinsic or extrinsic pathways. The differentials for this pattern of abnormalities include vitamin K-dependent coagulopathy, and isolated deficiencies of FX, FV, or FII. Definitive diagnosis is based on the specific finding of low FX coagulant activity (FX:C). Acquired FX deficiency has been reported in people with systemic amyloidosis, attributed to adsorption of FX onto amyloid fibrils. This phenomenon has not been documented in animals.

Fresh frozen plasma and cryosupernatant supply FX (see Chapter 96). After initial control of active hemor-

rhage, subsequent transfusions can be given at relatively long (q12–24 hours) interval due to the long half-life of FX of 40 hours.

Mis-sense and nonsense mutations are the most common mutation-type causing human FX deficiency; however, specific FX mutations have not yet been discovered in animals. The trait demonstrates a recessive inheritance pattern in people and animals. Familial testing in dogs and cats revealed severe deficiency (FX:C < 10%) in clinically affected patients and moderate factor deficiencies (20–50% FX:C) in obligate carrier parents.

Factor XI Deficiency

Thrombin activates FXI to form FXIa in the early initiation phase of coagulation. This reaction takes place most efficiently on the surface of activated platelets, where FXIa then generates FIXa and promotes assembly of the tenase coagulation complex (see Chapter 84).

Factor XI deficiency has been identified in people, dogs, cattle, and a DSH cat (Table 86.1; OMIM 264900).^{18,19,24} The defect is clinically variable in all species, but typically causes a mild bleeding tendency. Spontaneous hemorrhage is rare and affected patients are often diagnosed because of prolonged hemorrhage or abnormal rebleeding after surgery or trauma. Clinical severity does not correlate as closely with residual factor activity as most other factor deficiencies; however, clinical expression of a bleeding tendency is more likely to occur in patients with FXI activities (FXI:C) below 30% of normal.

Factor XI deficiency causes specific prolongation of the aPTT, with normal PT, TCT and fibrinogen (Table 86.1). Hemophilia A and B cause the same pattern of screening test abnormalities; however, specific coagulation factor analyses define isolated FXI deficiency (see Chapter 138).

Fresh frozen plasma and cryosupernatant supply FXI (see Chapter 96). Transfusion is usually required only for support of surgical or post-traumatic hemostasis. After an initial preoperative or loading transfusion, FXI half-life of approximately 50 hours allows repeat transfusions to be given at 12–24 hour intervals. Although their use has not been reported in animals, antifibrinolytic agents and topical fibrin sealants have been used successfully in human FXI deficient patients (see Chapter 90).

An exonic SINE insertion has been identified in FXI deficient Kerry Blue terriers and insertions in exons 3 and 9 have been described in FXI deficient Holstein and Japanese cattle, respectively.^{19,23} Homozygous cattle have marked FXI deficiency (FXI:C < 5%), whereas affected Kerry Blue terriers and DSH cats have relatively moderate reduction with FXI:C of approximately 2–15%.

Factor XII and Contact Factor Deficiencies

The contact group of coagulation factors includes FXII, prekallikrein (PK; Fletcher factor) and high molecular

weight kininogen (HMWK). Contact factors interact in vitro to initiate coagulation on artificial surfaces through activation of FXI. However, these factors are not required for fibrin clot formation in vivo and deficiency of these factors does not cause a bleeding diathesis (see Chapter 84). The physiologic activities of the contact group factors are now believed to include regulation of blood pressure, cellular proliferation, angiogenesis, and inflammation. Early reports of FXII deficiency in people suggested an increased risk of systemic thrombosis; however, a causal relationship has not been confirmed.

In addition to human beings, deficiencies of the contact pathway factors have been identified in dogs, cats, and horses (Table 86.1; OMIM 234000,229000,228960).^{10,12,17,25} Factor XII deficiency, in particular, is a common factor deficiency found in DSH and purebred cats.⁴

Contact pathway deficiencies are characterized by markedly prolonged aPTT (often >120 seconds), with normal PT, TCT, and fibrinogen. A prolonged aPTT that shortens by increasing activation time is a common characteristic of PK-deficient plasmas. Specific deficiencies of the contact group factors can be diagnosed based on functional assays of FXII, PK, and HMWK (see Chapter 138). Due to its high prevalence, FXII deficiency should be considered a likely cause of specific prolongation of aPTT in cats. FXII deficient cats do not require specific therapy. Long aPTT is a persistent in vitro finding but is not an indication for transfusion of any contact factor deficient patients.

Contact factor deficiencies are recessive traits; however, causative mutations have not yet been identified in animals. Cats homozygous for FXII deficiency have residual FXII:C values of approximately 2–10%, whereas heterozygous carriers generally have moderate reduction in activity with residual FXII:C from 20% to 40%.¹⁷

Combined Deficiencies of the Vitamin K-Dependent Factors

Factors II, VII, IX, and X are serine protease enzymes that require post-translational γ -carboxylation in order to form active complexes with their coenzymes and substrates. Defects in the hepatic vitamin K reductase or carboxylase enzymes impair vitamin K recycling and cause coagulopathy due to a persistent vitamin K deficiency state (see Chapter 84).

Familial combined deficiency of FII, VII, IX, and X is a rare recessive trait in people and has been identified in cats, sheep, and recently in Labrador retrievers (Table 86.1; OMIM 277450 and 607473).^{15,20,22} Clinical signs range from moderate to severe coagulopathy, with reports of spontaneous hematoma formation, prolonged hemorrhage after surgery, and some fatal hemorrhage.

Combined deficiency of the vitamin K-dependent factors causes prolongation of the aPTT and PT (and PIVKA) screening tests, but does not affect TCT and fibrinogen. Specific coagulation factor assays confirm deficiencies of FII, VII, IX, X, with normal activities of

the non-vitamin K-dependent factors. Acquired vitamin K-deficiency is common (see Chapter 85); therefore initial diagnostic work-up should rule out exposure to vitamin K antagonists, and liver disease or malabsorptive disorders. After exclusion of acquired disorders, specific analyses of hepatic microsomal vitamin K epoxide reductase (VKOR) and carboxylase enzyme activities further define a hereditary trait.

Patients with severe hemorrhage at presentation may require transfusion for rapid replacement of active factors (see Chapter 96). After an initial lag-time of 1–2 days, case reports describe normalization of aPTT and PT and control of clinical signs of hemorrhage in response to oral vitamin K₁ at approximately 2.5 mg/kg per day. Long-term maintenance of hemostasis has been reported at reduced daily dosages (e.g. 1 mg/kg/day) given at 3 day intervals.

Familial studies to date in all species reveal a recessive inheritance and expression pattern. The defective hepatic enzyme in cats and sheep is γ -glutamyl carboxylase; however, causative mutations in the corresponding genes have not yet been identified.

Unrecognized Factor Deficiencies

Hereditary deficiencies of FV, FXIII, and antiplasmin have been reported as rare hemorrhagic defects in people, with an estimated case incidence of <1 per million (OMIM 612309; 134570; 262850). The traits are autosomal recessive and cause clinically severe bleeding diatheses. Factor V deficiency causes prolongation of the aPTT and PT screening tests; however, identification of FXIII and antiplasmin deficiencies requires specific functional analyses of the corresponding proteins. While not yet recognized in animals, these defects should be included in the differential for severe hereditary coagulopathy after ruling out more common disorders.

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ON-LINE RESOURCES

1. National Institutes of Health. OMIM and OMIA. <http://www.ncbi.nlm.nih.gov/sites/gquery>
2. PennGenn Laboratories. Mutation detection tests for FVII and FXI deficiency. <http://research.vet.upenn.edu/PennGenHome/tabid/91/Default.aspx>
3. Comparative Coagulation Laboratory. Animal coagulation factor assays and genetic tests for hemophilia. <http://www.diaglab.vet.cornell.edu/coag/>

Thrombotic Disorders

SUSAN G. HACKNER and BARBARA DALLAP SCHAER

Pathophysiology	Antithrombin
Thrombotic Disorders	D-Dimer
Canine Thrombosis	Thrombin-Antithrombin Complex
Feline Thrombosis	Thrombin Generating Potential
Equine Thrombosis	Thromboelastography
Hypercoagulable Syndromes	Platelet Function Analysis
Protein-losing nephropathy	Platelet aggregometry
Neoplasia	Flow cytometry
Immune-mediated hemolytic anemia	Automated Platelet-sizing Parameters
Acute Pancreatic necrosis	Clinical Diagnosis of Thrombosis
Hypercortisolism	Pulmonary Thromboembolism
Cardiac Disease	Initial assessment
Atherosclerosis	Ancillary and definitive diagnostic tests
Sepsis	Aortic Thromboembolism
Diabetes mellitus	Feline aortic thromboembolism
Laboratory Tests of Hypercoagulability	Canine aortic thromboembolism
Coagulation profile	

Acronyms and Abbreviations

ADP, adenosine diphosphate; APC, activated protein C; aPTT, activated partial thromboplastin time; AT, anti-thrombin; ATE, aortic thromboembolism; CM, cardiomyopathy; COX, cyclo-oxygenase; CP, cancer procoagulant; CT, computerized tomography; DVT, deep vein thrombosis; ETP, endogenous thrombin potential; HAC, hyper-adrenocorticism; HCM, hypertrophic cardiomyopathy; IMHA, immune-mediated hemolytic anemia; NO, nitric oxide; MPC, mean platelet component concentration; MPCDW, mean platelet component distribution width; PAF, platelet activating factor; PAI-1, plasminogen activator inhibitor-1; PGL₂, prostacyclin; PLE, protein-losing enteropathy; PLN, protein-losing nephropathy; PT, prothrombin time; PTE, pulmonary thromboembolism; TAT, thrombin-antithrombin complex; TE, thromboembolism; TEG, thromboelastography; TF, tissue factor; TFPI, tissue factor pathway inhibitor; TNF, tumor necrosis factor; TxA₂, thromboxane A₂; VEGF, vascular endothelial growth factor; VWF, von Willebrand factor.

Thrombosis is the partial or complete obstruction of a blood vessel by a thrombus, or blood clot. The term thromboembolism (TE) refers collectively to obstruction from local thrombus formation (primary thrombosis) or translocation from a distant site (embolism). These events are difficult to differentiate clinically and produce indistinguishable pathophysiologic consequences. In addition to mechanical obstruction, thrombi release humoral factors and stimulate neurogenic reflexes. The signs of TE range from clinically inapparent events to organ failure and death, depending on the site and extent of vascular occlusion, collateral circulation, and the compensatory reserves of affected organs.

In spite of growing veterinary awareness, current TE management guidelines are based on incomplete information. Most recommendations are extrapolated from human trials, experimental models, and results of small veterinary studies, case series, and anecdotal reports.

PATHOPHYSIOLOGY

Virchow's triad describes three major factors promoting TE: endothelial injury, blood flow abnormalities (turbulence or stasis), and hypercoagulability.⁵ A single factor may predominate (Table 87.1); however, combined

abnormalities act synergistically to overcome hemostatic balance in most diseases. New models of cell-based hemostasis (see Chapter 84) and vascular bed-specific hemostasis⁵⁴ describe the complex interplay among cellular and plasma factors in various thrombotic syndromes.

In contrast to physiologic hemostasis where net forces favor blood flow, hypercoagulability refers to an imbalanced, prothrombotic state.^{3,7} This imbalance can be mediated by platelet hyper-reactivity, excessive activation of coagulation, deficiencies of natural anticoagulants, and/or hypofibrinolysis (Table 87.1).

Platelet hyper-reactivity results from increased platelet agonist activity or decreased agonist modulation (see Section VI). Factors that promote platelet activation include direct stimulation by inflammatory cytokines and tumor cells, cytokine or cell-mediated release of platelet agonists, and hypoalbuminemia-mediated upregulation of thromboxane A₂ (TxA₂).^{14,41} Platelet inhibition is attenuated by endothelial damage which decreases availability of prostacyclin (PGI₂), nitrous oxide (NO), and ADPase. Antiplatelet drugs may influence cyclo-oxygenase (COX) and membrane receptor activity.⁴⁶

Excessive activation of coagulation generally results from increased expression and/or exposure of tissue

factor (TF) (see Chapter 88). Endotoxin and inflammatory cytokines, particularly interleukin-6 (IL-6), induce TF expression on endothelial cells, macrophages, and cell-derived microparticles, and endothelial cell disruption exposes extravascular TF to the vascular space.³⁶ Some malignant cell-types express TF constitutively, and some produce a protease, cancer procoagulant (CP), that directly activates factor X.⁷

Lack of the physiologic anticoagulants antithrombin (AT), protein C, and tissue factor pathway inhibitor (TFPI) cause or contribute to hypercoagulability. Although numerous hereditary anticoagulant deficiency syndromes (thrombophilias) are recognized in people,^{3,10} anticoagulant deficiencies in animals are acquired disorders. Acquired AT deficiency is recognized as a consequence of decreased hepatic synthesis, increased loss, or excess consumption. Hepatic failure rarely leads to thrombosis because concomitant coagulation factor deficiencies favor hemorrhage.^{3,71} In contrast, increased glomerular permeability in protein-losing nephropathies (PLNs) permits selective loss of low molecular weight plasma proteins, such as albumin and AT, thereby producing a hypercoagulable state.^{9,59} Pathologic AT consumption occurs in conditions of increased thrombin generation, such as disseminated intravascular coagulation (DIC) or massive TE. Drugs (e.g. l-asparaginase and estrogen) are also associated with low plasma AT.³⁰

Protein C has a major anticoagulant effect at the endothelial cell surface and also modulates inflammation and endothelial cell apoptosis.¹⁶ A mutation in the human factor V gene (Factor V_{Leiden}) renders the protein resistant to activated protein C (APC) inhibition.^{3,10} This mutation is among the most common hereditary risk factors for deep vein thrombosis (DVT) in people of West European ancestry; however, a comparable syndrome has not been identified in animals. Acquired protein C deficiencies develop in association with sepsis, malignancy, hepatic or cardiac failure, pancreatitis, DIC, colic, laminitis and postoperatively.^{2,12,16,65} Low protein C activity appears to be a biomarker of hepatic synthetic capacity and portosystemic shunting in dogs, albeit with no observed increase in TE.⁶⁷ In contrast, recurrent TE developed in a foal with primary protein C deficiency.¹⁵

Tissue factor pathway inhibitor, synthesized by endothelial cells and platelets, regulates initiation of coagulation by inhibiting the factor VIIa-TF complex. Acquired TFPI deficiency has been related to hypercholesterolemia, and may represent a risk factor for arterial and venous TE.³

Hypofibrinolysis, primarily mediated by upregulation of plasminogen activator inhibitor (PAI-1) has also been implicated in the pathogenesis of hypercoagulability.^{3,10,25} As an acute phase protein, PAI-1 levels increase in association with inflammation and PAI-1 complexed with subendothelial fibronectin has inhibitory action on APC generation. High PAI-1 concentration has been associated with increased TE risk in people with atherosclerosis, sepsis, diabetes mellitus, post-trauma and surgery.²⁵ Although fibrinolytic defects

TABLE 87.1 Virchow's Triad Categorization of Prothrombotic Forces

Virchow's Triad Criteria	Forces
Vascular injury	Trauma Catheterization Inflammation Neoplastic invasion Parasitic damage Plaque deposition, atherosclerosis, amyloidosis
Stasis or turbulent flow	Hypoperfusion, hypovolemia, cardiac insufficiency, distributive Blood vessel compression Immobility Hyperviscosity, dehydration, polycythemia, leukemia, hyperglobulinemia, hyperfibrinogenemia Decreased RBC deformability, spherocytosis
Hypercoagulability	Platelet hyperaggregability, increased activation, decreased negative modulation Excessive activation of coagulation Decreased natural anticoagulant activity, antithrombin deficiency, (activated) protein C deficiency, TFPI deficiency ^a Hypofibrinolysis, decreased plasminogen activators, increased plasminogen activator inhibitors

^aTFPI, tissue factor pathway inhibitor.

are relatively ill-defined in animals, hypoplasminogenemia has been reported in traumatized dogs and in horses with strangulating obstructions,^{43,52} and increased PAI-1 and α 2-antiplasmin activities have been reported in septic foals.²

The concept of vascular-bed-specific hemostasis explains the observed variability in sites of TE in patients with systemic hypercoagulability.⁵⁴ This model refers to the uneven expression and local regulation of endothelial cell-derived prothrombotic and antithrombotic activities. Regional vascular beds, therefore, are nonuniformly affected by hypercoagulability in different disease syndromes, which in turn produces a variable tissue distribution of thrombus formation.

THROMBOTIC DISORDERS

Thromboembolic disease, traditionally classified as arterial or venous based on vessel of origin, is the leading cause of death in industrialized societies.^{1,3,71} Arterial TE is seen primarily in people with atherosclerosis, and manifests as acute coronary syndromes (myocardial infarction, angina) or cerebrovascular dysfunction (stroke, transient ischemic attack). Venous TE occurs secondary to congenital or systemic disease, and predominantly affects the deep veins in the extremities (DVT) and pulmonary vasculature (pulmonary thromboembolism; PTE).^{64,71} In contrast, animal TE typically

involves venous and/or arterial vessels with lower frequency than human TE.

Canine Thrombosis

Necropsy studies, case series, and case reports describe numerous conditions in dogs associated with TE.^{8,26,34,50,69,70} The most commonly reported include PLN, neoplasia, immune-mediated hemolytic anemia (IMHA), necrotizing pancreatitis, hyperadrenocorticism (HAC), and corticosteroid therapy (Table 87.2). Approximately 60% of PTE cases in retrospective studies have multiple predisposing conditions, suggesting a cumulative risk of TE. Canine thrombotic syndromes typically cause arterial and venous TE, with multi-organ involvement. Notable exceptions include TE restricted to the arterial vasculature in dogs with atherosclerosis^{27,69} and the propensity for PTE in dogs with IMHA.^{8,34,51}

Feline Thrombosis

Although overall TE frequency appears lower, feline thrombotic disorders are similar to those of dogs and people (Table 87.3).^{35,49,55,57} Aortic TE secondary to cardiomyopathy is by far the most common feline thrombotic syndrome (described in more detail below). Venous TE, albeit rare, has been reported in cats with neoplasia, cardiac disease, and complex, multisystemic disease.

TABLE 87.2 Canine Thrombotic Syndromes

Condition	Sites of thromboembolism										
	Pulmonary	Portal	Hepatic venous	Vena caval	Splenic	Renal	Mesenteric	Aortic/iliac	Myocardial	Cerebrovascular	Deep dermal
Protein-losing nephropathy	X	X	X	X	X	X	X	X	X	X	
Neoplasia	X	X		X	X			X	X	X	
IMHA	X	X	X	X	X	X			X		
Necrotizing pancreatitis	X	X			X			X	X		
Hyperadrenocorticism	X				X			X			
Corticosteroid therapy	X	X		X	X			X		?	
Infective endocarditis	X		X		X	X		X	X	X	X
Dirofilariasis	X				X			X		X	X
Miscellaneous cardiac disease ^a	X		X	X	X			X		X	
Atherosclerosis								X		X	
Sepsis	X	X		X	X				X	X	
Diabetes mellitus	X							X			
Protein-losing enteropathy								X			
Trauma									X	X	
Surgical procedures	X				X						
Parvoviral enteritis					X						
Spirocerca lupi								X			

^aMiscellaneous cardiac disease includes: valvular disorders, cardiomyopathy, myocardial failure, and anatomic anomalies of the atrium and vena cava.

TABLE 87.3 Feline Thrombotic Syndromes

Condition	Sites of thromboembolism										
	Pulmonary	Portal	Hepatic venous	Vena caval	Splenic	Renal	Mesenteric	Aortic/iliac	Myocardial	Cerebrovascular	Deep dermal
Neoplasia	X	X						X			
Cardiomyopathy	X					X	X	X		X	
Necrotizing pancreatitis	X	X									
IMHA	X										
Corticosteroid therapy	X										
Sepsis/SIRS	X										
Protein-losing nephropathy	X										
Protein-losing enteropathy	X										
Hepatic lipidosis	X										
Feline infectious peritonitis (FIP)	X										
Bacterial pneumonia	X										
Encephalitis	X										
Heartworm disease	X										
Congenital portosystemic shunt		X									

TABLE 87.4 Equine Thrombotic Syndromes

Condition	Sites of thromboembolism											
	Pulmonary	Jugular vein catheter site	Portal	Hepatic	Vena caval	Splenic	Renal	Mesenteric	Aortic/iliac	Myocardial	Cerebrovascular	Peripheral vasculature
Neoplasia			X									
Cardiac				X			X			X	X	
Immune-mediated hemolytic anemia (toxicosis)	X						X					
Sepsis/SIRS	X	X					X		X			X
Severe gastrointestinal disorders (endotoxemia)	X	X		X			X	X			X	X
Bacterial pneumonia	X	X										
Presumed verminous arteritis								X	X	X		

Equine Thrombosis

Vascular injury is a defined (or presumptive) cause of equine large and small vessel thrombosis (Table 87.4). Jugular vein catheter thrombosis and thrombophlebitis are relatively common, and reported risk factors include endotoxemia, hypoproteinemia, salmonellosis, and anti-diarrheal and anti-ulcer therapy.¹³ Parasite-induced intestinal vasculopathy and secondary TE are considered causes of acute and chronic colic, although the true incidence of this syndrome is not well-defined. Similarly,

distal limb thrombosis in foals and aortic and iliac TE, manifested as hind limb lameness, are presumed sequelae of parasitic endovascular migration. Distal limb thrombosis has also been reported secondary to sepsis and gastrointestinal inflammation. Horses are susceptible to infectious agents causing small vessel TE, including panarteritis due to equine viral arteritis and mycotic vasculitis due to disseminated aspergillosis (see Chapter 89). Equine hypercoagulability syndromes, characterized by multiorgan TE, have also been reported. Severe gastrointestinal disease and resultant endotoxemia are

often associated with renal microvascular TE. Microvascular thrombosis in the dermal lamina may play a role in the etiopathogenesis of carbohydrate-induced laminitis. As in dogs and cats, PTE is likely under-recognized in horses and its incidence rate is unknown. In a small case series, endotoxemia-induced hypercoagulability was considered an underlying cause of equine PTE, and previous case reports describe toxin-induced hemolysis and pneumonia as primary disorders in horses with PTE.⁴⁵ Cardiac disease is a relatively uncommon cause of TE in horses.

Hypercoagulable Syndromes

Protein-losing Nephropathy

Nephrotic syndrome is a well-recognized hypercoagulable state, with reported systemic thrombosis rates of 40% in people.⁵⁹ Molecular markers of thrombin activation and platelet aggregation increase with even modest urinary protein loss. Hypoalbuminemia (i.e. serum albumin concentration below 2.0 g/dL) provides a surrogate measure for TE risk.⁴² Protein-losing nephropathy is commonly associated with venous and arterial TE in dogs.^{9,22–24,26,34,50,70} In one study, TE was documented in 26% of dogs with PLN that died or were euthanized,⁹ however, relative TE risks have not been defined for specific renal disorders.

Urinary AT loss plays a major role in mediating the hypercoagulability of PLN.²³ Human patients experience thrombotic complications when plasma AT falls below 50–70%.⁵⁹ Concomitant hyperfibrinogenemia, hypercholesterolemia, high factor VIII, and hypofibrinolysis (due to high PAI-1) are believed to contribute to TE risk. Hypoalbuminemia is associated with enhanced platelet aggregability in dogs and people, and platelet reactivity correlates with proteinuria in people.^{23,59} Volume depletion, venous stasis, and immune complex activation may also promote TE secondary to PLN.

Neoplasia

Thrombosis accounts for significant morbidity and mortality in human and veterinary cancer patients, with high incidence associated with some tumor types, e.g. acute leukemias and solid tumors.^{7,22,33,34,49,53} Multiple mechanisms promote TE, including release of procoagulant substances and inflammatory cytokines by malignant cells, and direct interaction among tumor cells, endothelial cells, platelets, and monocytes (see Chapter 88).^{7,14} Human and animal tumor tissues demonstrate constitutive TF expression and release of cancer procoagulant (CP). Acquired APC resistance and anticoagulant deficiencies further exacerbate prothrombotic forces in some neoplastic disorders. Nonspecific contributory factors include vascular invasion by tumor cells, immobility, venous catheterization, corticosteroid therapy, surgery and chemotherapy.¹⁴

Enhanced or impaired fibrinolysis may develop secondary to neoplasia, with resultant signs of hemorrhage or thrombosis. The balance between plasminogen

activators and their inhibitors may also play a role in tumor metastasis. Tumor cell production of the proinflammatory cytokines tumor necrosis factor (TNF) and interleukin-1 β induce endothelial cell TF and PAI-1 expression, and downregulate the expression of thrombomodulin. Tumor cells also release vascular endothelial growth factor (VEGF), which induces macrophage expression of TF, increases microvascular permeability, and promotes tumor angiogenesis. Tumor cells, cell extracts, and membrane fragments promote platelet aggregation *in vitro*, and tumor cell injections cause *in vivo* platelet aggregation in experimental animals.^{7,14}

Immune-mediated Hemolytic Anemia

Venous TE is a major cause of canine IMHA mortality, with reported incidence at necropsy ranging from 30% to 80%.^{8,39,58} Hyperbilirubinemia, hypoalbuminemia, and severe thrombocytopenia are consistently associated with TE; however, other factors such as transfusion have shown variable association across studies. Laboratory evidence of hypercoagulability is well documented in IMHA case series; the most common abnormalities include hyperfibrinogenemia, high plasma D-dimer concentration, and low AT.⁵⁸ Although inflammatory mediators likely activate coagulation,³⁶ the pathogenesis of IMHA-associated TE is not fully elucidated. Proposed mechanisms include hemolysis mediated platelet hyper-reactivity and the presence of antiphospholipid antibodies.^{58,72}

Acute Pancreatic Necrosis

Thrombosis, correlated with disease severity, is common in people with acute pancreatitis.²⁸ Pancreatitis is also among the diseases associated with venous and arterial TE in dogs, and venous TE in cats.^{34,53,57,69,70} Experimental studies of pancreatitis in animals demonstrate platelet hyperaggregability, hyperfibrinogenemia, increased PAI-1 activity, and decreased t-PA activity.¹⁸ Pathogenic mechanisms of hypercoagulability include the release of TF and inflammatory cytokines from damaged pancreatic tissue.^{28,44} Pancreatitis also induces phospholipase A₂, thereby increasing platelet activating factor (PAF) and the production of cytokines (e.g. IL-1, IL-6, and TNF). Platelet reactivity may be further enhanced by high plasma free fatty acids and hypoalbuminemia, which inhibit synthesis of PGI₂ and promote TxA₂ release, respectively.

Hypercortisolism

Hyperadrenocorticism and corticosteroid therapy are considered risk factors for TE in dogs.^{6,34,69,70} Although systemic hypertension correlates with TE risk for people with Cushing's disease, the pathogenesis of TE in animals with hypercortisolism remains ill-defined, and a relationship between TE and dose or duration of corticosteroid therapy has not been established. Defining pathogenesis is difficult because Cushing's disease or steroid therapy are common in patients having other

potentially prothrombotic conditions, such as PLN, pancreatitis, diabetes mellitus, neoplasia, and IMHA. Postulated mechanisms for steroid-mediated hypercoagulability include hypercholesterolemia, hypofibrinolysis (due to increased PAI-1 and α -2 antiplasmin), hyperfibrinogenemia, and high coagulation factors.^{17,30}

Cardiac Disease

Infective endocarditis and heartworm disease are the most common cardiac disorders associated with TE in dogs.^{26,34,63} These disorders disrupt the endothelium and alter rheology, thereby promoting thrombus formation. In addition, exaggerated platelet aggregation and secretion response have been demonstrated in heartworm infected dogs.⁴ Although overt TE is rarely reported in dogs with congestive heart failure or valvular disorders, plasma indices of hypercoagulability (i.e. hyperfibrinogenemia, high thrombin-antithrombin complex (TAT) concentrations, and low AT and APC activities) have been described in dogs with cardiac disease.⁶⁵

Cardiomyopathy (CM), particularly hypertrophic CM (HCM), is the most common cause of arterial TE in cats. Reported incidence rates of TE secondary to CM range from 12% to 50%.^{55,60} Thrombus formation typically occurs in the left atrium leading to aortic TE (ATE).^{35,55,60} Cats with left atrial enlargement and/or echocardiographic “swirling” of blood are considered more likely to develop TE; however, relative risks associated with these findings are not defined. The pathogenesis of cardiomyopathy-induced TE is likely multifactorial. Possibilities include endothelial disruption, blood turbulence or stasis, platelet hyper-reactivity, and endothelial dysfunction secondary to metabolic imbalance.^{40,60} Hemostatic abnormalities indicative of systemic hypercoagulability, including high TAT and D-dimer concentration have recently been reported in case series of HCM cats with and without TE.⁶²

Atherosclerosis

Myocardial infarction and other arterial TE are well-recognized consequences of atherosclerosis in people. Although relatively uncommon in animals, atherosclerosis associated with aortic and cerebrovascular TE has been reported in hypothyroid dogs.^{27,69} Mechanisms promoting TE include endothelial disruption by atherosclerotic plaques, hypercholesterolemia, and a prothrombotic lipoprotein profile (see Chapter 89).

Sepsis

Sepsis and the systemic inflammatory response syndrome (SIRS) are common triggers of microvascular thrombosis and DIC; however, macrothrombus formation with venous and arterial TE have also been reported in animals with inflammatory syndromes.^{11,52,57,69,70} The pathogenesis of thrombosis in these disorders is complex, mediated by inflammatory cytokines, hemostatic proteins, leukocytes, platelets, and endothelial cells (see Chapter 88).³⁶

Diabetes Mellitus

Thromboembolic disease is an uncommon complication of diabetes mellitus in dogs, and most cases have concomitant conditions that might exacerbate or induce a thrombotic tendency.^{24,50,69} Platelet hyper-reactivity and refractoriness to inhibition are believed to play a role in diabetes-associated TE.⁴⁶ Loss of insulin’s modulating effects on platelet activation and decreased endothelial synthesis of PGI₂ and NO combine to potentiate platelet response. Hyperglycemia also correlates with increased plasma PAI-1, resulting in decreased fibrinolysis.

LABORATORY TESTS OF HYPERCOAGULABILITY

The complex pathogenesis of hypercoagulability and its associated disease states complicates laboratory diagnosis of TE. Testing strategies include assays designed to measure “global” or net hemostatic forces and scoring systems based on routine tests and clinical parameters (see Chapter 88). The diagnostic utility of new and traditional tests is based on relating test results with clinical outcomes.

Coagulation Profile

The routine coagulogram (activated partial thromboplastin time [aPTT], prothrombin time [PT], and fibrinogen) screens for risk of hemorrhage due to coagulation factor deficiency (see Chapter 138). Although variable shortening or prolongation of aPTT and PT are often found in patients with TE, these changes are non-specific and rarely aid in diagnosis or management. Hyperfibrinogenemia, however, is an independent risk factor for TE in people⁷¹ and is a common finding in canine IMHA, feline CM, and sepsis.^{2,52,58,62} The predictive value of fibrinogen determinations for animal TE has yet to be established.

Antithrombin

Antithrombin activity relates to TE risk in conditions such as PLN, where AT deficiency plays a major role in pathogenesis of hypercoagulability.⁹ While risk of TE has been attributed to AT values below 60%,²³ there is little empiric data in animals at this time to define a specific AT threshold.

D-Dimer

D-Dimer is a distinct fibrin degradation product formed by plasmin proteolysis of cross-linked fibrin (see Chapter 138). Quantitative D-dimer assays are sensitive (but nonspecific) tests with proven diagnostic utility for ruling out PTE, and for predicting mortality in human thrombotic syndromes.^{61,64} D-dimer assays are now used for screening animals, with studies in progress to better define their clinical utility (see Chapter 88).^{47,62,65}

Thrombin-Antithrombin Complex

Free thrombin in plasma is neutralized by the formation of an irreversible, 1:1 complex with the anticoagulant protein, AT. The plasma concentration of the resultant thrombin-antithrombin complex (TAT) is an indirect measure of *in vivo* thrombin generation. The finding of high TAT is evidence of an active hypercoagulable state and TAT analyses have been used recently to characterize thrombotic syndromes in dogs, cats, and horses (see Chapter 88).^{62,65}

Thrombin Generating Potential

The endogenous thrombin potential (ETP) is a new assay technique to directly measure the *in vitro* capacity of plasma to generate thrombin over time. The ETP is considered a “global test” whose parameters reflect the net effect of coagulation pathway activators and inhibitors. Studies of the diagnostic utility of ETP for various thrombotic syndromes in people are currently underway.⁶⁸

Thromboelastography

Thromboelastography (TEG) measures the viscoelastic properties of a nascent fibrin clot in whole blood.²⁰ The point-of-care test generates qualitative tracings and numeric values depicting clot strength and stability, and the dynamics of clot formation and breakdown (Fig. 87.1). Thromboelastography has been used in human patients to test for hypo- and hypercoagulability, to monitor anticoagulant effects, to guide transfusion therapy, and to predict postoperative TE. Thromboelastographic features of hypercoagulability have been described in dogs with neoplasia, DIC, IMHA, parvoviral enteritis, and following deracoxib therapy.^{32,43,73} Evaluation of the TEG and other viscoelastic monitors for clinical management of thrombotic syndromes in animals is currently underway.¹¹

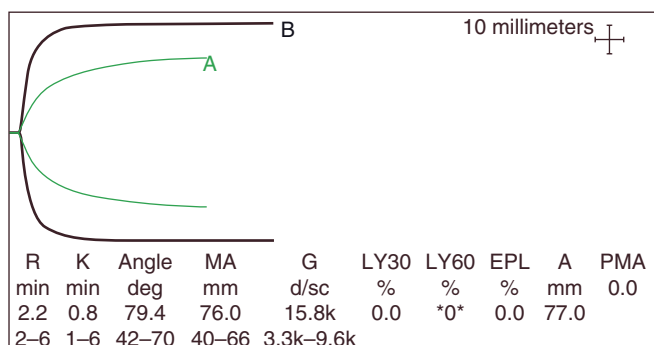


FIGURE 87.1 Tissue-factor activated TEG of a healthy dog and a dog with hyperadrenocorticism. (A) Healthy dog demonstrating normal TEG tracing. (B) Dog with hyperadrenocorticism demonstrating parameters of hypercoagulability: shortened clot formation time (K), increased angle (α), maximum amplitude (MA) and clot elasticity (G) values.

Platelet Function Analyses

Laboratory assessment of platelet function is complex, including measurements of platelet size, ability to aggregate, and expression of various activation markers. (see Chapter 142).

Platelet Aggregometry

Measurement of platelet aggregation response to agonists (e.g. ADP, collagen) is the traditional gold standard of *in vitro* platelet function testing.⁴¹ While aggregometry has proven utility in the diagnosis of platelet “hyporeactivity” in people and animals, aggregation studies are generally not useful for clinical characterization of TE, and have limited research applications in gauging response to anti-platelet agents.

Flow Cytometry

Flow cytometry enables the detection of specific aspects of platelet function, including platelet size, granularity, expression of activation markers and presence of bound fibrinogen.^{45,72} Exposure of P-selectin (an alpha granule protein) on the platelet outer membrane surface is an indicator of platelet degranulation and marker of platelet activation (see Chapter 137). High platelet P-selectin expression has been demonstrated in clinical studies of dogs with inflammatory disease and IMHA.^{45,72}

Automated Platelet-sizing Parameters

Automated hematology analyzers measure several parameters of platelet function.^{37,45} Mean platelet component concentration (MPC) is a measure of refractive index, which is linearly related to platelet density. Activated (i.e. degranulated) platelets have relatively low density compared to non-degranulated platelets. Mean platelet component distribution width (MPCDW) measures the uniformity in activation status. Mean platelet component correlated with P-selectin expression in dogs with inflammatory disease;⁴⁵ however, additional studies are needed to clarify diagnostic utility of MPC for TE.

CLINICAL DIAGNOSIS OF THROMBOSIS

Pulmonary Thromboembolism

The diagnosis of PTE is challenging because clinical signs mimic other conditions (e.g. pneumonia, cardiac disease), results of routine tests are often nonspecific, and definitive tests are invasive, expensive, and/or not widely available. An index of suspicion is critical; PTE should be considered for any patient with an acute onset of respiratory signs, particularly in the presence of underlying hypercoagulable states.

Initial Assessment

Clinical signs of PTE reflect the magnitude of respiratory and cardiac compromise.^{34,48,49,64} Common signs

include acute dyspnea, tachypnea, and depression. Severe signs range from cough, hemoptysis, and cyanosis, to collapse, shock, and sudden death. Thoracic auscultation may reveal tachycardia, split heart sounds, and adventitious lung sounds. Patients in left heart failure have signs of hypoperfusion.

Diagnostic evaluation should include thoracic radiographs, arterial blood gas analyses, and routine hemogram and biochemistry profiles. Common radiographic abnormalities include pulmonary infiltrates (alveolar, interstitial, or mixed pattern) with indistinct borders in the right and caudal lobes.^{19,26} Some cases have lobar radio-opacities and wedge-shaped densities with the apex toward the pulmonary hilus. Radiolucent vascular filling defects distal to the thrombotic occlusion are best visualized on ventrodorsal or dorsoventral views. (Fig. 87.2). Main pulmonary artery segment enlargement, attenuation of lobar vessels, generalized cardiomegaly, and pleural effusion may also occur.^{19,34} Disproportionately severe respiratory signs with mild radiographic abnormalities are suggestive of PTE.

Arterial blood gas analyses, although non-pathognomic, provide diagnostic and management information.^{26,34,64} An increased alveolar-arterial oxygen tension gradient (A-a gradient) reflects ventilation-perfusion (V/Q) mismatch and is almost invariably present, whereas hypoxemia occurs in approximately 80% of dogs. Hypoxemia reflects the severity of vascular occlusion and usually responds to supplemental oxygen. Diminished oxygen response occurs with extreme V/Q mismatch and intrapulmonary shunting. Hypercapnia may occur with severe compromise.

Abnormal hemogram and biochemistry profiles generally reflect inflammation, hypoxemia, or stress. Test results may suggest additional work-up to identify hypercoagulable states. Hyperfibrinogenemia, low AT



FIGURE 87.2 Vento-dorsal survey thoracic radiograph of a dog with PTE. Note oligemia of the right caudal lung fields.

and TEG parameters consistent with hypercoagulability further support the presence of PTE and should prompt investigation for underlying disorders (Tables 87.1, 87.2, 87.3, 87.4).

Ancillary and Definitive Diagnostic Tests

D-Dimer is used as an exclusionary test of PTE in people; low pretest PTE probability and low D-dimer values obviate the need for imaging studies.^{61,64} Although test algorithms have not yet been validated for animals, case series have generally shown high sensitivity (80–90%) for D-dimer assays in veterinary patients with PTE and other thrombotic disorders.^{47,62} High D-dimer is also found in animals with neoplasia, hepatic disease, renal or cardiac failure, internal hemorrhage, and after surgical procedures. Specificity for TE increases with markedly elevated D-dimer values (i.e. >1,000 ng/mL).⁴⁷

Echocardiography is a valuable diagnostic tool for PTE in people, and is a rapid and non-invasive means to rule out primary cardiac disease in dyspneic patients.^{21,64} Abnormalities typical of human PTE include right ventricular dilation and hypokinesis, pulmonary arterial hypertension, tricuspid regurgitation, and paradoxical septal wall motion. Regional hypokinesis sparing the ventricular apex (McConnell sign) is a specific finding.³⁸ Reported PTE-associated echocardiographic abnormalities in animals include dilation of the right atrium, ventricle, and pulmonary artery, pulmonary hypertension, paradoxical septal wall motion, and visualization of a thrombus.^{29,33,49}

Pulmonary angiography, scintigraphy, and helical computerized tomography (CT) are definitive tests for PTE.^{31,61} Selective pulmonary angiography, performed by direct injection of iodinated dye into the pulmonary artery was the early gold standard.^{61,64} Intraluminal filling defects, abrupt termination of pulmonary arteries, and complete absence of arterial branches are diagnostic for PTE. Additional supportive findings include regional loss of vascularity, asymmetric blood flow, tortuous pulmonary arteries, and abrupt tapering of peripheral vessels. A negative selective arteriogram excludes clinically significant PTE. The invasive procedure requires general anesthesia, thus constituting a safety risk for compromised patients. Nonselective pulmonary angiography, performed by injecting contrast media into the jugular vein or right side of the heart, is a simpler and safer technique that does not require general anesthesia. Nonselective angiography, however, is relatively insensitive and difficult to interpret due to dilution of contrast medium by venous blood and the superimposition of vascular structures.

Pulmonary scintigraphy is a sensitive and specific test of PTE; however, its requirement for radiolabeling and specialized equipment limit its routine veterinary use.³¹ The procedure involves sequential scans that assess pulmonary blood flow (perfusion scan) and air flow (ventilation scan) to identify regions of ventilated lung lacking perfusion (i.e. V/Q mismatch). Perfusion scans are performed by injecting technetium-labeled macroaggregated albumin (^{99m}Tc-MAA) into a central vein. Distribution is proportional to blood flow; inho-

ogeneous distribution is consistent with abnormal perfusion. Perfusion scanning is contraindicated in patients with severe pulmonary hypertension because ^{99m}Tc -MAA particles can occlude capillaries and cause right-sided heart failure. A normal perfusion scan excludes PTE; however, abnormal scans can result from nonthrombotic conditions (e.g. pneumonia, edema, contusions, obstructive pulmonary disease, atelectasis). In contrast to PTE, nonthrombotic conditions are associated with decreased regional ventilation. For this reason, interpretation of abnormal perfusion scans requires assessment of pulmonary ventilation. Ventilation scans are performed by the administration of a nontoxic radioactive gas, such as technetium-labeled diethylene-tiamene-penta-acetic acid (^{99m}Tc -DTPA), via closed ventilation circuit.³¹ Hypoventilated lung regions appear photopenic. While PTE disrupts regional blood flow, ventilation scans are typically normal. Severely dyspneic animals may be unable to undergo ventilation scans. In these cases, an absence of radiographic abnormalities in regions of abnormal perfusion can be considered evidence of V/Q mismatch, compatible with PTE.

Helical, or spiral, computed tomographic angiography (CTPA) has largely replaced angiography and scintigraphy for the definitive diagnosis of PTE in people.^{61,64,66} This rapid, noninvasive technique enables high resolution visualization of the pulmonary vasculature. Moreover, CTPA can define nonthrombotic pulmonary disease and is ideal for unstable patients. Advanced, "multislice" CTPA techniques enable quality images in awake or minimally sedated patients. The technique is extremely accurate in detecting thrombi in the main, lobar or segmental arteries. Recent studies describe CTPA to visualize the pulmonary vasculature and detect PTE in dogs.⁶⁶

Aortic Thromboembolism

Feline Aortic Thromboembolism

Aortic TE is a common and devastating condition in cats, with reported survival-to-discharge rates of approximately 30%.^{35,55,60} The inciting cause is usually HCM, with additional cases attributed to other cardiac disease, or rarely neoplasia.^{57,60} Thrombi typically form in the left ventricle with embolization to the aortic trifurcation ("saddle thrombus") causing blood flow obstruction of both hind limbs. Less frequently, emboli lodge proximal to the renal arteries, or obstruct a single iliac or brachial vessel. Acute obstruction of the left ventricle or proximal aorta may cause sudden death. The consequences of ATE include physical occlusion of the vessel and embolus-induced vasoconstriction that disrupts collateral flow. Serotonin and TxA_2 have been implicated as mediators of this intense vasoconstriction.⁵⁶ Aortic occlusion increases left ventricular afterload, which may precipitate heart failure.

The diagnosis of ATE is usually based on characteristic history and clinical signs.⁶⁰ Affected cats develop acute pain, vocalization, and loss of motor function.

Physical exam may reveal swollen, tense hind limb musculature, cool extremities, pale or dark purple pads and nailbeds, and weak or absent femoral pulses. Varying sites of occlusion produce variable signs such as paresis (or paralysis), loss of sensation, or renal dysfunction or failure. At presentation many cats are dyspneic due to heart failure, with heart murmurs and/or gallop rhythms. More specific assessment of limb perfusion can be performed with color flow Doppler imaging or by cutting a nail back to the "quick" and observing the presence or absence of normal blood color and flow. Angiographic and nuclear imaging are definitive PTE tests; however, the risks of these invasive procedures are rarely justified.

Cats with suspect or confirmed ATE should undergo cardiac evaluation (i.e. echocardiography, electrocardiography, thoracic radiography). Up to 90% of cats have cardiomegaly, and 50–70% have evidence of heart failure.³⁵ Electrocardiographic abnormalities include left ventricular enlargement and conduction abnormalities, left anterior fascicular block, and ventricular and supraventricular premature beats. Biochemical profiles often reveal hyperglycemia and other nonspecific abnormalities reflecting muscle ischemia and stress. Uremia may develop in patients with renal vascular TE. In the absence of cardiac disease, diagnostic efforts should be directed toward identification of other potential hypercoagulable states, especially neoplasia.

Canine Aortic Thromboembolism

Although canine ATE is relatively uncommon, it has been reported in association with cardiac disease (infective endocarditis, heartworm, cardiomyopathy, and valvular disorders), PLN, PLE, neoplasia, hyperadrenocorticism, atherosclerosis, and localized vascular injury (e.g. *Spirocerca lupi*).^{29,63,69}

Clinical signs depend on TE site, the extent and rapidity of occlusion, and the degree of collateral circulation.²² Dogs with underlying cardiac disease tend to have acute onset and severe signs resembling those of ATE in cats. Hypercoagulable syndromes generally cause local thrombosis with more insidious onset. In these cases, exercise intolerance, ill-defined pain and mild locomotor deficits or ataxia may develop. Femoral pulses may be absent, weak, or asymmetric. Prognosis generally depends on the underlying condition.

Definitive diagnosis of ATE is achieved via imaging.^{29,33} Abdominal ultrasonography is most commonly employed and is usually reliable, especially when combined with color flow Doppler assessment. Additional diagnostic techniques include conventional and radio-nuclide angiography, magnetic resonance imaging, thermography, and Doppler device to assess blood flow to the affected limbs. Since canine ATE is rarely a primary disease, the diagnosis should always prompt a thorough evaluation for underlying disorders. Biochemical profiles of affected dogs often reveal high creatinine kinase and aspartate aminotransferase; however, other biochemical abnormalities may provide more specific indication of a primary disease process.

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Disseminated Intravascular Coagulation

TRACY STOKOL

Causes

Pathogenesis

Initiation

Progression/Dissemination

Amplification of thrombin production

Role of phosphatidylserine

Dissemination of coagulation

Fibrinolysis

Perpetuation of DIC

Inflammatory response

Endothelial cell dysfunction

Other factors

Diagnosis of DIC

Overt DIC

Clinical diagnosis

Laboratory criteria of overt DIC in humans

Scoring System for Overt DIC in Humans

Non-Overt DIC

Treatment of DIC

Replacement of Coagulation Factors and Platelets

Anticoagulant Therapy

Replacement of Inhibitors and Anti-inflammatory Therapy

Acronyms and Abbreviations

APC, activated protein C; aPTT, activated partial thromboplastin time; AT, antithrombin; CT, computerized tomography; DIC, disseminated intravascular coagulation; EC, endothelial cells; FDP, fibrin(ogen) degradation product; FV/FVa, factor V/activated factor V; FVII/FVIIa, factor VII/activated factor VII; FVIII/FVIIIa, factor VIII/activated factor VIII; FIX/FIXa, factor IX/activated factor IX; FX/FXa, factor X/activated factor X; FXI/FXIa, factor XI/activated factor XI; FXIII, factor XIII; IMHA, immune-mediated hemolytic anemia; ISTH, International Society of Thrombosis and Hemostasis; MP, microparticle; PAR, protease-activated receptor; PCI, protein C inhibitor; PS, phosphatidylserine; PT, prothrombin time; RBC, red blood cell; TAFI, thrombin-activatable fibrinolysis inhibitor; TAT, thrombin-antithrombin complexes; TCT, thrombin clotting time; TF, tissue factor; TFPI, tissue factor pathway inhibitor.

Disseminated intravascular coagulation (DIC) describes a complex, dynamic state of hemostatic imbalance resulting in thrombus formation throughout the microvasculature. Many different primary disorders trigger DIC, and its clinical and laboratory manifestations vary among patients and change over time, complicating its recognition and treatment.⁶ Hematologists have recently developed guidelines delineating distinct phases of overt and non-overt DIC, with the goals of standardizing terminology and improving clinical diagnosis (see Table 88.1).^{54,57}

The International Society of Thrombosis and Hemostasis (ISTH) has proposed a consensus definition of DIC as follows: "An acquired syndrome characterized by the intravascular activation of coagulation with loss of localization resulting from different causes. It can originate from and cause damage to the microvas-

culature, which if sufficiently severe, can produce organ dysfunction."⁵⁴

PRIMARY DISEASE CONDITIONS

An underlying disease process always initiates DIC; it is not a primary disorder (see Table 88.2).

DIC has been documented in most domestic animals, with the exception of camelids. In dogs and cats, neoplasia and systemic inflammation (e.g. sepsis, pancreatitis, immune-mediated hemolytic anemia [IMHA]) are the most common initiating diseases.^{13,17,46,48,49,56} Endotoxemia (secondary to gastrointestinal disorders) and sepsis are the main causes of DIC in adult horses and neonatal foals, respectively.^{3,15,50} Similarly, DIC is primarily due to endotoxemia or sepsis in ruminants.^{26,39}

TABLE 88.1 Characterization of Overt and Non-Overt DIC

	Overt DIC	Non-Overt DIC
Alternate names and subtypes	Uncompensated DIC Acute DIC Fulminant DIC Consumptive coagulopathy Hypocoagulable DIC	Compensated DIC Chronic DIC Subclinical DIC Pre-DIC
Pathophysiology	Decompensated hemostasis with fibrin deposition throughout the microvasculature. Platelet and factor depletion may develop	Procoagulant excess opposed by coagulation inhibitors, partial compensation modulates fibrin deposition
Clinical features	Primary inciting disease Some or all of the following: Systemic thrombosis, PTE, MODS ^a Hemorrhage from multiple sites	Primary inciting disease Subclinical or tissue thrombosis
Screening test abnormalities	Some or all of the following: Thrombocytopenia, prolonged clotting times (aPTT, PT, TCT), hypofibrinogenemia, low AT, high FDP and D-dimer	No screening test abnormalities and/or progressive fall in platelet count and increase in clotting times, high FDP and D-dimer
Tests of pre-thrombotic state	Not applicable	Byproducts of thrombin generation or thrombin activity: Prothrombin fragment 1+2, TAT complex, APC-PCI complex, Fibrinopeptide A & B Kinetics of thrombin or fibrin generation: Endogenous thrombin potential Thromboelastography

^aMODS, multiple organ dysfunction syndrome; PTE, pulmonary thromboembolism.

PATHOGENESIS

Over the past decade, our understanding of DIC has evolved from recognition of a severe hemorrhagic disorder to appreciation of DIC as a disease continuum. The process is initiated by inappropriate activation of hemostasis, at first kept in check by natural inhibitors. If regulatory mechanisms become overwhelmed, DIC progresses to a full-blown decompensated disorder characterized by systemic thrombosis and ultimately, a consumptive coagulopathy.^{6,57} Much of the current knowledge of DIC pathogenesis is derived from experimental models of sepsis-induced DIC.^{31,54} Although disease-dependent differences influence the initiation and clinical course of DIC, the consistent pathogenic mechanism remains the generation of excessive thrombin through dysregulated activation of coagulation.⁵⁴ For ease of understanding, the DIC continuum can be separated into three stages: initiation, progression/dissemination, and perpetuation.^{19,54,57}

Initiation

Since DIC represents normal hemostasis gone awry, the same triggering event underlies DIC and physiologic hemostasis, i.e. exposure of tissue factor (see Chapter 84).²⁴ Tissue factor (TF, tissue thromboplastin, factor III) is a transmembrane protein whose expression is normally restricted to interstitial perivascular fibroblasts. Under physiologic conditions, exposure of TF to

its plasma ligand, factor VII (FVII), occurs only at focal sites of endothelial cell injury.²³

In DIC triggered by massive tissue trauma, widespread endothelial injury exposes supraphysiologic amounts of extravascular TF. In conditions such as viral infections, intravascular hemolysis, and vasculitis, generalized endothelial injury exposes extravascular TF and/or induces TF expression on damaged endothelium. Inflammatory cytokines also contribute to TF expression in these latter disorders (see Table 88.2).¹⁹

In the absence of endothelial injury, DIC may be initiated by aberrant expression of TF on the surface of intravascular cells, particularly monocytes and tumor cells (see Table 88.2). The pathogenesis of DIC in sepsis involves up-regulation of TF expression on monocytes (and possibly endothelial cells) by proinflammatory cytokines (Fig. 88.1).^{19,25}

Although cancer cells have many ways of activating hemostasis, some types of neoplasms constitutively express high levels of TF thereby promoting cancer-associated DIC.²¹ TF-independent mechanisms of initiating DIC also exist (see Table 88.2). For example, proteases identified in erythrocyte membranes, tumor cells, and snake venoms can directly activate coagulation factors.^{28,47,63}

The development of DIC depends not only on the location and severity of the inciting stimulus, but on the ability of naturally occurring inhibitors to regulate the procoagulant hemostatic response. In physiologic hemostasis, the TF-FVIIa-FXa complex is rapidly neu-

TABLE 88.2 Inciting Diseases and Pathophysiologic Mechanisms of DIC in Animals

Disease Categories and Specific Disorders	Potential Mechanisms
Infections Sepsis (Gram positive and negative bacteria), viruses, protozoa (<i>Babesia</i>), parasites (<i>Angiostrongylus</i>), rickettsia (Rocky Mountain spotted fever)	Cytokine-induced TF expression on monocytes (and EC) Endothelial injury Exposure of extravascular TF or induction of TF on EC
Neoplasia Solid tumors (hemangiosarcoma, mammary cancer) Hematopoietic malignancy (lymphoma, acute leukemia)	Expression of TF on cancer cells Constitutive or induced by hypoxia, cytokines, or apoptosis Induction of TF expression Tumor-secreted cytokines act on monocytes, fibroblasts, and EC Shedding of TF-bearing microparticles from tumor cells, hematopoietic cells, other cell types Expression/secretion of procoagulants Cancer procoagulant (a vitamin K-dependent cysteine protease) mucin, up-regulation of factor V receptor, factor XIII-like activity, platelet activators Chemotherapy-induced changes Tumor lysis, myelotoxicity
Inflammation/necrosis Trauma, IMHA, pancreatitis, heat stroke, hepatitis, vasculitis, gastric dilatation-volvulus, strangulating obstructions and inflammatory gastrointestinal disorders	Inflammatory cytokines EC injury Tissue injury/necrosis/apoptosis Exposure of TF; shedding of PS-enriched MPs by apoptotic cells Release of procoagulant proteases (e.g. trypsin)
Intravascular hemolysis IMHA, acute transfusion reactions, insect/snake bites	EC injury Erythrocyte procoagulant activity RBC membrane-associated elastase, shedding of PS-enriched MPs
Envenomation	Snake venom proteases Direct coagulation factor activation and fibrinogen cleavage, phospholipase-induced tissue injury/cell lysis

tralized by tissue factor pathway inhibitor (TFPI). TFPI is primarily expressed on endothelial cells, with its activity enhanced by cell-surface heparin and heparin-like glycosaminoglycans.³² In the process of DIC, TFPI is rendered ineffective through a number of mechanisms, including cleavage of TFPI by granulocytic elastases,

cytokine-mediated suppression of TFPI expression, and generation of excess TF-FVIIa that overwhelms TFPI's inhibitory capacity.^{22,43}

Progression/Dissemination

Amplification of Thrombin Production

Thrombin is pivotal to DIC progression (Figs. 88.1 and 88.2). In a positive feedback cycle, trace amounts of generated thrombin are amplified by intrinsic pathway reactions to produce a massive burst of thrombin.^{24,57} The thrombin burst cleaves soluble fibrinogen to form fibrin polymers, and ultimately the cross-linked fibrin clot. Thrombin simultaneously inhibits fibrin degradation via thrombin-activatable fibrinolysis inhibitor (TAFI). In early DIC, as in physiologic hemostasis, the generation of thrombin and its actions are opposed by the plasma anticoagulants, antithrombin (AT) and protein C, and the endothelial cell surface receptor, thrombomodulin (see Fig. 88.2 and Chapter 84). This phase of systemically activated coagulation restrained by natural anticoagulants is referred to as “non-overt” DIC, a state of stressed, but compensated hemostasis (Table 88.1 and Fig. 88.1).^{54,55} Patients with non-overt DIC are hypercoagulable, i.e. at risk for widespread microvascular fibrin deposition.

Role of Phosphatidylserine

Coagulation is a membrane-anchored process involving assembly of coagulation factor complexes on the surface of cells expressing the negatively charged phospholipid, phosphatidylserine (PS; see Chapter 84).²⁴ Resting cells restrict PS to the inner leaflet of their cell membrane; however, this asymmetry is lost when cells are activated, undergo apoptosis, or lyse.³⁰ During physiologic hemostasis, activated platelets provide the membrane-surface PS to support formation of coagulation complexes; hence PS is referred to as “platelet factor 3” or platelet phospholipid. In the course of DIC, cell-surface PS may be expressed by a variety of activated or injured cell types, by membrane-derived microparticles (MPs), and circulating lipoproteins (native very low density lipoproteins and oxidized low density lipoproteins).^{14,20,45} Microparticles are tiny (<1 μm) PS-enriched membrane vesicles liberated from the surface of activated monocytes (Fig. 88.1), platelets, injured or activated endothelial cells, apoptotic cells, and cancer cells.²⁰ Monocyte-derived MPs also express TF. Microparticles bring various hemostatic components into close proximity and provide a large surface area for assembly of active coagulation complexes. Due to their small size, MPs are slowly cleared from the circulation and are believed to play a role in promoting systemic thrombin formation.

Dissemination of Coagulation

As the underlying disease progresses and natural coagulation inhibitors become overwhelmed, TF expression

FIGURE 88.1 The DIC continuum in sepsis. Inflammatory cytokines initially induce TF expression on monocytes. Monocyte-surface TF, FVIIa, and FXa form an active complex which generates thrombin. In early, non-overt DIC, inhibitors control the activated hemostatic system. If inhibitory mechanisms become overwhelmed, then thrombin amplifies its own production, generating systemic thrombin. MPs shed from thrombin-activated cells provide a surface that supports systemic coagulation complex assembly. In the perpetuation phase, thrombin signaling through PARs activates monocytes and endothelial cells which potentiates the inflammatory response. The depletion of anti-inflammatory coagulation inhibitors further facilitates inflammation thereby maintaining the sepsis-DIC cycle.

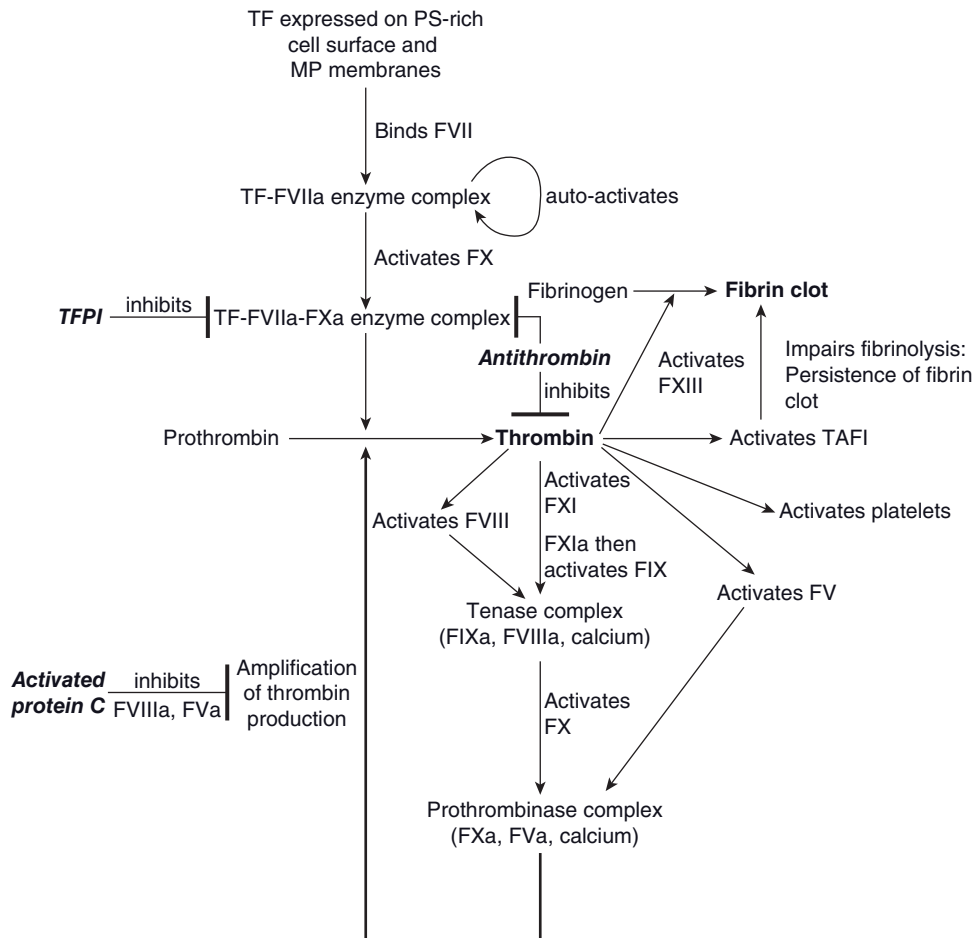
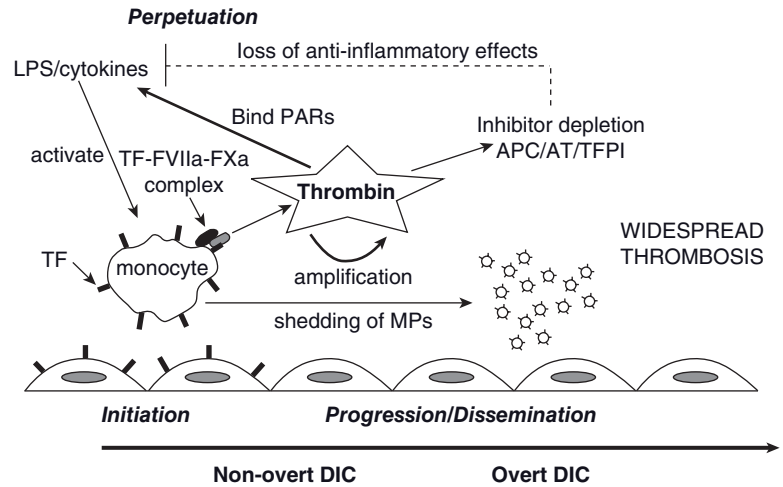


FIGURE 88.2 The pivotal role of thrombin in coagulation. Coagulation is triggered in DIC via the extrinsic pathway, when FVII binds TF, forming an autocatalytic complex. This binds to and activates FX; the TF-FVIIa-FXa complex then converts small amounts of prothrombin to thrombin. Thrombin amplifies its own production via the intrinsic pathway through FXI, which in turn activates FIX. Thrombin also activates the cofactors, FVIII and FV. Factor IXa forms a potent enzymatic (“tenase”) complex with FVIIIa and calcium, which activates FX. FXa, with FVa as a cofactor and calcium (“prothrombinase” complex) generates an explosive burst of thrombin. Thrombin thus formed cleaves fibrinogen to fibrin and activates factor XIII (FXIII) (which cross-links fibrin), platelets, and TAFI. TAFI inhibits fibrinolysis, preventing clot breakdown. TFPI inhibits the TF-FVIIa-FXa complex, antithrombin inactivates thrombin and other proteases, and activated protein C inhibits FVIIIa and FVa (which decelerates thrombin generation). The entire series of enzymatic reactions occurs on the surface of PS-expressing cell or MP membranes.

TABLE 88.3 Mechanisms of Anticoagulant Deficiency in DIC

Mechanism	Primary Condition
Decreased hepatic synthesis AT, protein C, protein S	Hepatic disease, inflammatory cytokines
Decreased protein C activation	Down-regulation of thrombomodulin by inflammatory cytokines
Decreased activity TFPI, AT, protein C, protein S	Decreased free protein S (due to increased C4-binding protein, an acute phase reactant protein), down-regulation of heparin-like glycosaminoglycans on endothelial cells, cleavage of endothelial protein C receptor
Increased consumption AT, protein C	Rapid clearance of TAT and activated protein C-protein C inhibitor complexes
Loss TFPI, AT, protein C, protein S	Vascular leakage
Degradation TFPI, AT, protein C, protein S	Proteases, e.g. neutrophil elastases

persists and thrombin generation proceeds unopposed (Table 88.3).

Thrombin-mediated recruitment and activation of platelets provides additional PS-bearing surfaces to support coagulation. Tissue factor- and PS-enriched MPs help amplify and disseminate the coagulation process. The unchecked and systemic generation of thrombin produces diffuse microvascular fibrin thrombi, the hallmark of overt DIC (Table 88.1 and Fig. 88.1).

Fibrinolysis

Under conditions of physiologic hemostasis, plasmin-mediated fibrinolysis is stimulated by the release of tissue plasminogen activator from damaged endothelial cells (see Chapter 84). Fibrinolysis is also stimulated by inflammation, via activation of the contact pathway and through direct elaboration of proteases by granulocytes, neoplastic cells, and snake venoms.^{10,31,36} Nevertheless, fibrinolysis is unable to maintain vascular patency in overt DIC, due in part to cytokine-induced endothelial cell secretion of plasminogen activator inhibitors, and activation of TAFI by thrombin.^{9,19}

Perpetuation of DIC

Inflammatory Response

A self-perpetuating cycle develops in DIC triggered by sepsis or non-septic inflammation (Fig. 88.1).^{31,57} Inflammation initiates coagulation via tissue injury, induced TF expression, and platelet and leukocyte activation and microvesiculation. Coagulation, in turn,

fuels inflammation, through the proinflammatory properties of active coagulation factors (e.g. thrombin, FXa, FVIIa).^{7,12,31} In addition to its central role in coagulation, thrombin binds to protease-activated receptors (PARs) on monocytes and endothelial cells, thereby stimulating cytokine secretion and adhesion molecule expression. Systemic depletion of anticoagulants results in the loss of the anti-inflammatory properties of coagulation inhibitors (particularly protein C and AT) and cell surface receptors (thrombomodulin and endothelial cell protein C receptor).^{31,33,44,57} Experimental studies of TF-induced DIC indicate that in the absence of inflammation, TF alone does not sustain the positive feedback cycle.⁴²

Endothelial Cell Dysfunction

Perturbed endothelial function (due to a primary disease or ensuing hypoxia) is another mechanism for perpetuating DIC.^{6,31} Endothelial dysfunction causes a loss of inhibition and a gain of proinflammatory and procoagulant properties. Healthy endothelial cells secrete potent platelet inhibitors (e.g. prostacyclin) and express receptors that modulate coagulation and inflammation. Upon injury, these functions are lost and endothelial cells may secrete inflammatory cytokines and platelet activating compounds.

Other Factors

The release of vasoactive peptides (e.g. bradykinin, endothelin) from damaged or thrombin-stimulated cells induces hemodynamic changes that often decrease tissue perfusion, thereby contributing to hypoxic injury.^{11,27} Ischemic and necrotic tissue injury, in turn, promotes inflammation and coagulation.³¹ Secondary acidosis inhibits platelet function and coagulation factor activity, furthering development of hemorrhagic DIC.³⁵

DIAGNOSIS OF DIC

Every animal with an inciting primary disease should be considered at risk for developing DIC (Table 88.2). Confirmation of DIC is challenging because animals may be examined at any point of the DIC continuum, clinical signs may be subtle or nonspecific, and individual laboratory tests are variably sensitive and specific for this syndrome. Histopathologic evidence of fibrin thrombi throughout the microvasculature in biopsy or necropsy specimens represents a “gold standard” test. Unfortunately, this test modality has low diagnostic utility because biopsy specimens are inconsistently obtained antemortem, and fibrin thrombi often lyse rapidly after death.^{40,52}

Current trends in human medicine shift the emphasis toward early detection of both non-overt and overt DIC.^{4,55,57} Studies of patients with sepsis reveal that antimicrobial therapy alone rarely halts progression of DIC, suggesting that improved clinical outcomes will require earlier and more directed therapeutic interventions.

The need for better strategies to diagnose early DIC is also recognized in veterinary medicine.^{5,16,61}

Overt DIC

Clinical Diagnosis

Documentation of a primary initiating disease is the minimum requirement for diagnosis of DIC in all species (Table 88.2). The term overt DIC refers to an active thrombotic syndrome, with fibrin deposition throughout multiple organs (e.g. heart, lungs, liver, kidney, central nervous system). Widespread microvascular thrombosis decreases blood flow to vital tissues thereby causing hypoxic injury, cell death, and organ failure; factors ultimately responsible for the high morbidity and mortality of DIC.^{5,6,15,57} Although large vessel thrombosis may be detected on physical examination, sophisticated imaging techniques such as helical computerized tomography (CT) and CT angiography are often the only means to identify medium and small vessel thrombosis and thromboembolism.⁵³ Since these imaging modalities are impractical for clinical veterinary practice, patients with primary inciting diseases should be monitored for the adverse effects of thrombosis on organ function, e.g. ventilation/perfusion mismatch, azotemia, hepatocellular injury or lactic acidosis. Indeed, azotemia and lactic acidosis were more severe in horses with acute colitis and laboratory criteria of DIC than those without these criteria. Furthermore, those horses in DIC were eight times more likely to die.¹⁵ While differentiating the effects of primary disease from DIC can be difficult, signs of progressive organ dysfunction in critical care patients should be considered potential evidence of thrombosis associated with overt DIC.

A subset of overt DIC patients have a fulminant disease course characterized by signs of shock (from diffuse thrombosis and release of vasoactive peptides) and/or active hemorrhage. The bleeding diathesis often manifests as one or more of the following signs: epistaxis, petechiae, bruising, prolonged bleeding after venipuncture or minor surgical procedures, spontaneous hematoma formation and body cavity hemorrhage. Hemorrhagic DIC develops frequently in dogs, but appears to be uncommon in horses and cats.^{3,15,17,50,56} Although severe signs at presentation often prompt laboratory investigation, screening tests to detect overt DIC should be performed in all patients with primary inciting disorders (Tables 88.1 and 88.2).

Laboratory Criteria of Overt DIC

Diagnosis of overt DIC relies upon identifying abnormalities in multiple tests, rather than a single pathognomonic sign or laboratory finding (Table 88.1).^{6,19} Traditional criteria for diagnosis of overt DIC in animals include a combination of two or more test abnormalities, specifically thrombocytopenia, prolonged coagulation times (prothrombin time [PT], activated partial thromboplastin time [aPTT], thrombin clotting time [TCT]), hypofibrinogenemia, low AT, and high fibrin(ogen) degradation product (FDPs) or D-dimer.^{3,13,15,17,48,50,56} The

apparent diagnostic utility of these tests varies depending on species and stage at presentation.

Mild to moderate thrombocytopenia is a consistent finding in dogs with overt DIC (75–100% sensitivity),^{5,48} but not in cats¹⁷ or horses (0–64% sensitivity).^{15,50} Overt DIC is therefore unlikely in dogs having stable platelet counts remaining within reference intervals. Of the routine coagulation screening tests, the aPTT appears to be more sensitive for detecting DIC in animals than the PT or TCT.^{15,17,46,48,50} Hypofibrinogenemia is generally an insensitive indicator of DIC because fibrinogen is an acute phase reactant protein and likely to be upregulated secondary to underlying inflammatory disorders.^{15,17,48,50} Indeed, the finding of normal fibrinogen values in a patient with active inflammation suggests ongoing consumption of fibrinogen in a DIC process. Low AT activity is one of the more sensitive tests for diagnosis of DIC in dogs (77–90%)^{5,46,48} and horses (89–93%).^{15,50} In cats with DIC, AT activities may acutely decrease; however, values rarely fall below reference intervals and may actually increase in some patients.^{8,56} In most diagnostic laboratories, D-dimer assays have replaced FDP testing for detecting fibrinolysis in DIC. D-dimer is a sensitive test for DIC in dogs (75–100%)^{41,46,49} but is less sensitive in horses (50%),⁵⁰ and has not been adequately evaluated in cats. Additional tests sometimes used to characterize DIC include blood smear examination for erythrocyte fragments, measurement of soluble fibrin monomer and individual coagulation factor activities (e.g. factor V).^{18,48}

Scoring System for Overt DIC in Humans

The DIC subcommittee of the ISTH recently developed a practical system for consistent diagnosis of overt DIC in people.⁵⁴ The system is applied to patients with an underlying disorder associated with DIC and derives a summary score from results of four readily available laboratory tests: platelet count, PT, fibrinogen, and fibrin-related markers (soluble fibrin, FDP or D-dimer; Fig. 88.3).

These laboratory tests were deliberately chosen because they are widely available and suitable for serial monitoring and uniform comparisons among treatment trials. The ISTH also emphasized that clinical trials should include consistent and relevant outcome measures, such as decreased fatality rates, rather than relying solely on the DIC test score.^{4,54} Following adoption of this recommendation, recent studies have used 28-day fatality rates as standard outcome measures to assess validity of the calculated DIC score.^{4,29,58,59} Results of these studies demonstrate strong correlation between overt DIC (i.e. ISTH score ≥ 5) and patient mortality. Inclusion of fibrinogen in several trials did not increase prognostic power of the score. The ISTH scoring system is still undergoing validation to further refine its diagnostic utility.

Non-Overt DIC

Non-overt DIC is characterized by an activated, but not overwhelmed, hemostatic system.^{19,55} Non-overt DIC

1. Risk assessment: Does the patient have an underlying disorder known to be associated with overt DIC?
If yes: proceed; If no: do not use this algorithm;
2. Order global coagulation tests (platelet count, prothrombin time (PT) fibrinogen, soluble fibrin monomers or fibrin degradation products).
3. Score global coagulation test results
 - Platelet count ($>100 \times 10^9/L = 0$; $<100 \times 10^9/L = 1$; $<50 \times 10^9/L = 2$)
 - Elevated fibrin-related marker (eg., D-dimer/fibrin degradation products) (no increase: 0; moderate increase: 2; strong increase: 3)*
 - Prolonged prothrombin time ($<3 \text{ sec} = 0$; $>3 \text{ sec but } <6 \text{ sec} = 1$; $>6 \text{ sec} = 2$)
 - Fibrinogen level ($>1.0 \text{ g/L} = 0$; $<1.0 \text{ g/L} = 1$)
4. Calculate score
5. If ≥ 5 : compatible with overt DIC; repeat scoring daily
If < 5 : suggestive (not affirmative) for non-overt DIC; repeat next 1-2 days

* Values corresponding to a moderate or strong increase are test- and laboratory-dependent.

FIGURE 88.3 ISTH scoring system for overt DIC in man. (Adapted from Taylor FB, Jr., Toh CH, Hoots WK, et al. Towards definition, clinical and laboratory criteria, and a scoring system for disseminated intravascular coagulation. *Thromb Haemostasis* 2001;86:1327–1330, with permission.)

also applies to the clinical designation of hypercoagulability or “chronic DIC” reflecting continuous low grade, but compensated, activation of coagulation (Table 88.1 and Fig. 88.1). Routine coagulation screening tests are insensitive to this phase because they detect coagulation factor deficiencies, rather than accelerated coagulation. Technically sophisticated assays that measure the rate and byproducts of thrombin generation, coagulation inhibitor complexes, and kinetics of fibrin formation provide mechanistic and prognostic information on non-overt DIC (Table 88.1).^{38,51,57} Unfortunately, these tests are not widely available for routine clinical diagnosis.

Thus, the ISTH has developed a second algorithm for the diagnosis of non-overt DIC primarily based on routine laboratory tests.⁵⁴ The scoring system requires serial testing to evaluate the dynamic nature of non-overt DIC and awards points for an underlying disease (Fig. 88.4).

The ISTH scheme for non-overt DIC has been modified and applied to critically ill dogs.⁶² In a preliminary study, the sensitivity of the modified scheme for diagnosing non-overt DIC and predicting 28-day mortality rates was compared to that of traditional criteria for diagnosing DIC (i.e. three or more abnormalities in hemostatic tests). More dogs were diagnosed with DIC using traditional criteria; however, the classification of non-overt DIC was more closely related to mortality.⁶² Further evaluation is needed to identify the optimum tests and test cut-offs to include in a DIC scoring scheme. Furthermore, due to species-differences in underlying diseases and diagnostic test sensitivity for DIC, it is unlikely that a single scoring system will apply across species. The strategy of serial monitoring of routine laboratory tests (e.g. platelet count, APTT, AT, D-dimer, fibrinogen) should be evaluated for clinical diagnosis of

non-overt DIC. Trends toward abnormal values would indicate hemostatic system decompensation and progression of DIC, even if absolute values remain within assay reference intervals. Point-of-care “global tests of hemostasis” such as thromboelastography may also prove useful for identifying animals in hypercoagulable phases of DIC.^{16,61}

Ultimately, there is a need to establish consensus guidelines for diagnosing non-overt and overt DIC in animals. This would serve as a basis for achieving consistency across studies to assess the impact of early diagnosis and various treatment protocols on defined clinical outcomes.

TREATMENT OF DIC

Affected animals should always be treated for their primary disease with the goal of breaking the DIC cycle. Supportive care aimed at alleviating metabolic/hemodynamic sequelae of DIC (shock, hypoperfusion and acidosis) helps minimize organ damage, inflammation, and continued activation of hemostasis. Effective treatment modalities beyond primary disease-specific and supportive care remain unproven. General treatment options include transfusion therapy and anticoagulant and anti-inflammatory drug therapy (see Chapters 90 and 96). Treatment recommendations are generally derived from the human literature and should be applied with the knowledge that they may not be suitable for animals, due to species-specific differences in hemostasis and drug pharmacokinetics and efficacy. Randomized, controlled clinical trials, with collaboration between multiple institutions or practices and consensus on testing criteria and a defined outcome (e.g. 28-day mortality), are urgently needed in veteri-

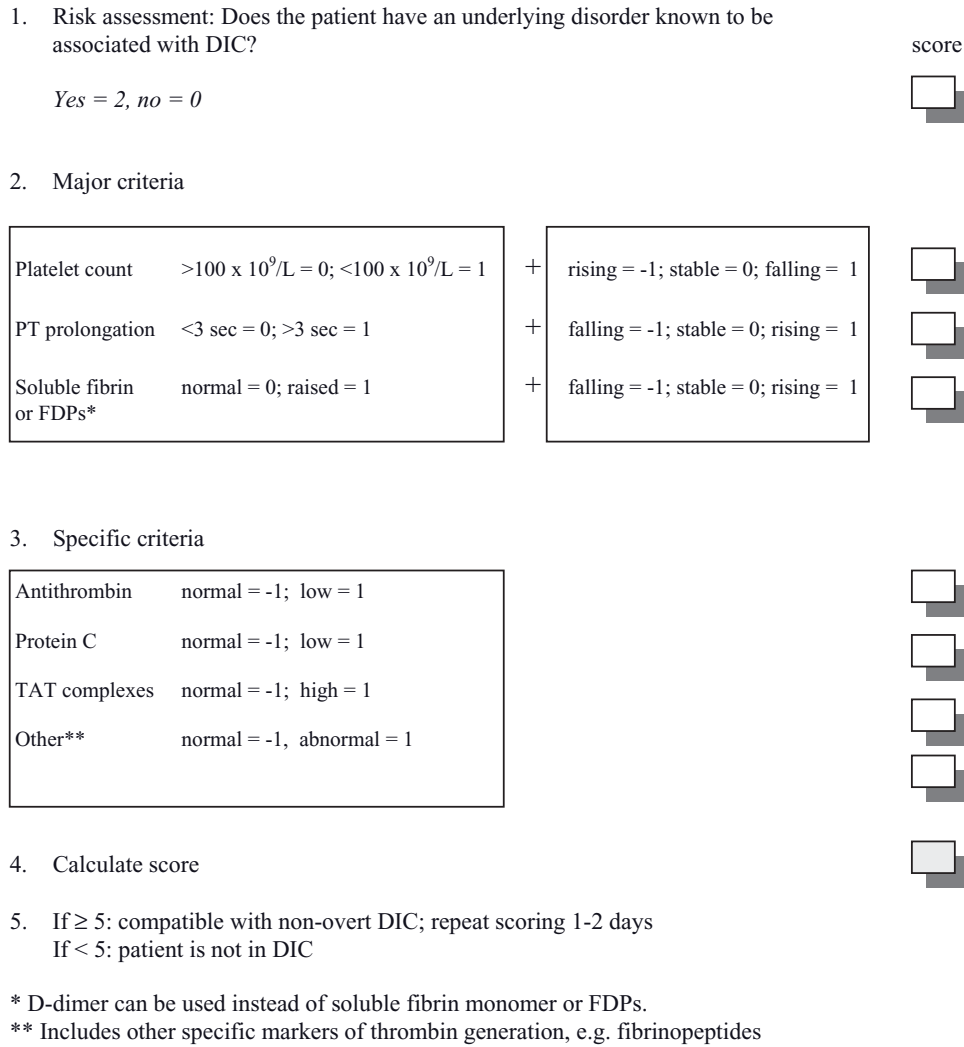


FIGURE 88.4 ISTH diagnostic algorithm for non-overt DIC in man. (Adapted from Taylor FB, Jr., Toh CH, Hoots WK, et al. Towards definition, clinical and laboratory criteria, and a scoring system for disseminated intravascular coagulation. *Thromb Haemostasis* 2001;86:1327–1330, with permission.)

nary medicine to identify safe and cost-effective DIC treatment strategies.

Replacement of Coagulation Factors and Platelets

The fear that transfusions “fuel the fire” of DIC is largely unfounded; however, transfusion therapy is generally restricted to actively bleeding patients in human studies. Component therapy, rather than whole blood, generally provides the most effective means of restoring adequate levels of factors or platelets to support hemostasis (see Chapters 96 and 97).

Anticoagulant Therapy

Although thrombosis underlies the morbidity and mortality of DIC, anticoagulant therapy, particularly heparin therapy, has not been proven to be a consistently effective therapy of overt DIC in people.¹⁹ More specific case selection for heparin therapy may improve

clinical outcomes;^{34,37} however, pending new information on therapeutic efficacy, heparin should be used with caution for treating DIC in animals (see Chapter 90). Recommendations for unfractionated and low molecular weight heparin (LMWH) therapy in animals have been adopted from humans. Several recent studies have shown that currently used dosing regimes for LMWH do not attain therapeutic anti-FXa levels or are ineffective at inhibiting coagulation.^{1,37}

Replacement of Inhibitors and Anti-inflammatory Therapy

Many human trials of the inhibitor concentrates, TFPI, AT and activated protein C (APC),^{2,19,29,60} have been performed with the goal of preventing DIC progression. Of these, administration of AT (without concurrent heparin)²⁹ and APC^{2,19} have shown some promise in reducing mortality in sepsis-associated DIC. Unfortunately, these concentrates are prohibitively expen-

sive, unavailable or unsuitable for use in animals.²⁵ With the recognition that many cases of DIC are initiated by inflammatory disorders, future studies will likely place greater emphasis on limiting inflammation in animals with DIC. It is possible that drug combinations (e.g. inhibitors with anticoagulants) or novel drugs aimed at modulating inflammation-coagulation interactions can ultimately improve clinical outcomes by breaking the DIC cycle.

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Vascular Diseases

SEAN P. MCDONOUGH

Congenital Anomalies and Malformations

Shunts

- Persistent ductus arteriosus
- Persistent ductus venosus
- Arteriovenous fistula

Telangiectasia

Varicose Veins

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- Equine herpesvirus-1
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Rocky Mountain Spotted Fever

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Immune-mediated Vasculitis

- Equine purpura hemorrhagica
- Canine juvenile polyarteritis syndrome

Paraneoplastic Vasculitis

Acronyms and Abbreviations

ADE, antibody dependent enhancement; AVF, arteriovenous fistula; CJPS, canine juvenile polyarteritis syndrome; DV, ductus venosus; EC, endothelial cell; EHV-1, equine herpesvirus-1; EVA, equine viral arteritis; FECV, feline enteric corona virus; FIPV, feline infectious peritonitis virus; FMD, fibromuscular dysplasia; IFN- γ , interferon-gamma; IL, interleukin; MCF, malignant catarrhal fever; PDA, persistent ductus arteriosus; SMC, smooth muscle cell; TNF- α , tumor necrosis factor-alpha.

Blood vessels contain three concentric layers: intima, media, and adventitia (Fig. 89.1). The intima is a single layer of endothelial cells (ECs) that rests on scant subendothelial connective tissue, separated from the media by the internal elastic membrane. The media is composed of smooth muscle cells (SMCs), collagen and elastin fibers, and fibroblasts. The outermost adventitia is a layer of dense fibroelastic connective tissue separated from the media of larger arteries by an external elastic membrane.

Hemodynamic stress evokes a variety of responses from all mural elements.^{10,15} Vascular injury induces intimal migration and proliferation of SMCs. Smooth muscle contraction regulates regional blood flow and pressure. ECs have a myriad of functions, including the transfer of molecules across vessel walls and the modulation of blood flow, vascular resistance, hormone metabolism, SMC growth, and inflammatory and immune response. Moreover, the prothrombotic and antithrombotic properties of ECs play a critical role in regulating hemostasis.

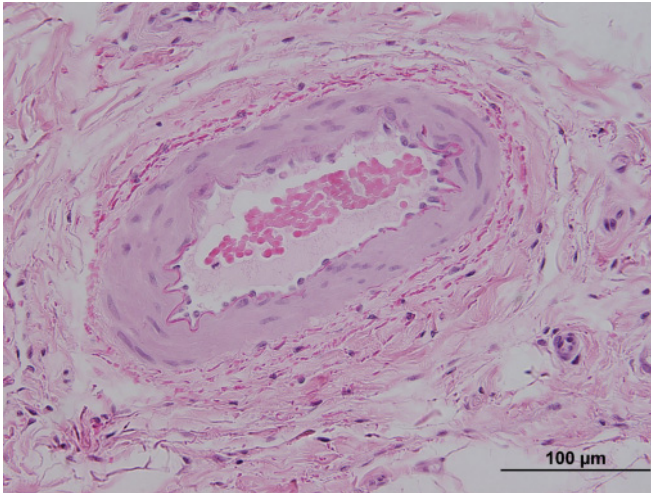


FIGURE 89.1 Cross section view of a normal muscular arteriole. A single layer of endothelial cells adjacent to the lumen is surrounded by smooth muscle cells and fibroblasts in the media, and an outermost loosely organized layer of adventitial cells. H&E stain; 40 \times .

CONGENITAL ANOMALIES AND MALFORMATIONS

Shunts

Persistent Ductus Arteriosus

The ductus arteriosus is the fetal vascular communication between the pulmonary artery and aorta. Hypoplasia or asymmetry of ductal SMCs and aorta-like elastic tissue in the ductus cause failure to contract properly after birth and persistence of a vascular lumen.² PDA occurs in all species, but is most common in dogs.

Persistent Ductus Venosus

The fetal ductus venosus (DV) allows umbilical and portal venous blood flow to bypass the liver.³² An abrupt decrease in blood flow and pressure in the umbilical sinus shortly after birth causes retraction and narrowing of the DV orifice, resulting in functional shunt closure. Permanent closure via connective tissue deposition starts within days after birth and is complete by 15–18 days in the dog. Persistent DV may cause hepatic encephalopathy, with lobular atrophy, reduplication of the hepatic arterioles, and unapparent portal veins in the hepatic triads.

Arteriovenous Fistula

Arteriovenous fistulas (AVFs) are direct arterial-venous communications without intervening capillary beds. AVFs may be congenital defects or develop from penetrating wounds, rupture of arterial aneurysms into adjacent veins, or inflammatory necrosis of vessel walls. AVFs consist of enlarged congested vessels with disorganized arterial walls and thin walled, dilated venous channels. AVFs typically cause signs of recurrent hem-

orrhage; however, large AVFs may induce high output heart failure, and AVF in the CNS may cause neurologic signs and acute death.¹¹

Telangiectasia

Telangiectasias are congenital or acquired exaggerations of preformed vessels. The condition is most common in the liver of cattle, characterized by increased deposition of basement membrane components and fibrosis resulting in hepatocyte atrophy and disruption of sinusoids.¹³

Varicose Veins

Varicosities, most common on the ovary and vulvar lips of horses, are dilated, tortuous veins produced by prolonged increased intraluminal pressure and loss of vessel wall support.

DEGENERATION OF ARTERIES

Atherosclerosis

Atherosclerosis is a chronic inflammatory response initiated by EC injury in the arterial wall, with progression mediated by macrophages, T-cells, SMCs, and the adjacent extracellular matrix.³⁴ Atherosclerosis is common in parrots and lesions occasionally develop in the coronary arteries of dogs with hypothyroidism or diabetes mellitus.⁹ In early lesions, lipid-laden macrophages, T-cells, and small amounts of extracellular lipid accumulate in fatty streaks. Over time, macrophage derived IL-1 and TNF- α recruit more leukocytes, produce toxic oxygen species, and elaborate growth factors that stimulate medial SMCs to migrate to the intima, and to divide and deposit extracellular matrix. Fatty streaks may evolve into atherosclerotic plaques that protrude into the vessel lumen causing ischemic damage. Focal disruption of the plaque exposes a central necrotic mass composed of lipid, cholesterol clefts, cellular debris, foam cells, fibrin, variably organized thrombi, and other plasma proteins. These substances are highly thrombogenic, resulting in thrombosis and embolization. Atrophy of the media underlying atherosclerotic plaques may lead to aneurysmal dilation and arterial rupture.

Vascular Amyloid

Amyloid is a pathologic protein formed from precursors such as serum amyloid-associated protein, immunoglobulin light chain, and apolipoprotein A. Abnormal folding of precursors leads to the formation of oligomers and fibrils that are eventually deposited as β -pleated sheets. Histologically, amyloid is an amorphous, acellular eosinophilic extracellular deposit. Amyloid shows a characteristic apple green birefringence when stained with Congo red and examined under polarized light. Vascular amyloid deposition in the media of splenic and cardiac vessels is a common incidental finding in aged dogs, occasionally associated with EC necrosis and obliteration of the vascular lumen. Cerebral amyloid

angiopathy in cortical and leptomeningeal vessels is common in aged dogs and cats due to the deposition of β -amyloid produced by SMCs. Apolipoprotein AI-derived amyloid has been described in pulmonary vessels of old dogs, perhaps due to aberrant metabolism or mutant forms of apolipoprotein AI.

Mineralization

Uremic Gastropathy

Uremic gastropathy occurs most often in dogs with renal disease and occasionally in cats and horses. Vascular damage, presumably induced by a poorly characterized toxic peptide, results in gastric mucosal edema, congestion, and hemorrhage.¹⁹ Medial necrosis, mineralization, and thrombosis of arterioles in the middle and deeper portions of the gastric mucosa may accompany EC injury. Despite the vasculopathy, gastric necrosis and ulceration rarely develop.

Medial Calcific Sclerosis

Medial calcific sclerosis (Monckeberg's arteriosclerosis) refers to flat plaques in the media of elastic arteries composed of calcium and phosphorus deposits. Osseous metaplasia with hematopoietic tissue in the intertrabecular spaces may develop at these sites.

Metastatic Mineralization

Metastatic mineralization, due to disturbance of calcium and phosphorus metabolism, occurs in vitamin D toxicity, secondary to chronic granulomatous diseases such as John's disease, and in herbivores that consume plants such as *Solanum malacoxylon* or *Cestrum diurnum* that contain 1,25-dihydroxycholecalciferol or a related compound.¹⁶

Arterial Ruptures

Trauma is the most common cause of arterial rupture in all species. Aortic rupture, from the level of the aortic valve to the brachiocephalic trunk, is best documented in horses²⁷ but the syndrome has been reported in camelids and woodchucks. Degenerative changes of the media, including cyst formation, fragmentation and mineralization of elastin fibers, and accumulation of myxomatous ground substance has been proposed as a risk factor for aortic rupture. Rapid death is usually due to hemopericardium; however, dissection into the myocardium may occur. Additional syndromes in horses include uterine artery rupture and mycotic ulceration of the internal carotid or maxillary artery. Experimental copper deficiency in pigs leads to rupture of the aorta due to deficiency of the copper-containing enzyme lysyl oxidase. Similarly, lathyrism due to β -aminopropionitrile inhibits lysyl oxidase.

Aneurysms

Aneurysms are localized abnormal dilations of a vessel. True aneurysms consist of multiple layers of the vessel

wall, while false aneurysms are hematomas (surrounded by fibrous connective tissue) that communicate with the vessel lumen. Abdominal artery aneurysms are common in Holstein cattle in the northeastern United States.¹²

HYPERTROPHY OF ARTERIES

Chronic Hypoxic Pulmonary Hypertension

High-altitude disease refers to a syndrome of heart failure in cattle and pigs secondary to chronic hypoxic pulmonary hypertension. The pulmonary vasculature of cattle has distinctive, well-developed media in small arteries and veins. After initial vasoconstriction, chronic hypoxia induces medial hypertrophy of the small pulmonary arteries and a functional obstruction. Hypertrophy and dilation of the right ventricle eventually lead to right heart failure with ventral edema (brisket disease).²² Some researchers propose that transforming growth factor- β 1 mediates variation among individuals in the response to hypoxia. This growth factor, elaborated by perivascular neutrophils and mononuclear cells of small pulmonary arteries, appears to stimulate differentiation of vascular fibroblasts into myofibroblasts, a crucial step in hypoxia-induced vascular remodeling.⁸

Hypertension

Systemic Hypertension

Systemic hypertension is most often reported in dogs and cats with chronic renal failure, diabetes mellitus, hyperthyroidism, hyperadrenocorticism, and chronic anemia.¹ Systemic and local hypertension is reported in horses and cattle with laminitis. Chronic renal disease is typically associated with hyperplastic arteriosclerosis, as interlobular and intralobular renal arteries and afferent arterioles thicken due to medial hypertrophy and adventitial fibrosis. Hyaline arteriosclerosis refers to the deposition of a homogeneous, pale pink hyaline material that obscures mural elements. These vascular changes occur frequently in the nodular arterioles of the spleen of dogs and pigs, and in the uterine and ovarian arteries of pregnant and post-partum animals. Similar changes, but of unknown significance, occur in the intramural coronary arteries and small meningeal and cerebral vessels of dogs. Arteriolar hyaline degeneration is seen in the heart of pigs with vitamin E/selenium deficiency (mulberry heart disease)²¹ and edema disease.

Pulmonary Hypertension

Pulmonary hypertension can either cause or result from pulmonary artery disease. Cardiac anomalies that lead to left to right shunting, hypoxic pulmonary vasoconstriction, and pulmonary thromboembolism may increase vascular resistance resulting in pulmonary arterial hypertension, whereas left-sided heart failure causes venous pulmonary hypertension and pulmonary edema. A secondary vasculitis, resulting from EC

degeneration and fibrinoid necrosis of the media, often accompanies the acute pulmonary hypertension of PDA. Chronic pulmonary hypertension leads to a spectrum of changes including isolated medial hypertrophy, medial hypertrophy with intimal thickening, and medial hypertrophy with intimal thickening and concurrent plexiform lesions (plexogenic pulmonary arteriopathy). Plexiform lesions, indicative of end-stage damage, consist of networks of small vessels spanning the lumen of thin-walled, dilated arteries.

Feline Pulmonary Artery Medial Hypertrophy

Pulmonary artery medial hypertrophy is a striking vascular lesion in cats.²³ The vascular changes, however, do not cause pulmonary hypertension or right ventricular hypertrophy. Medial changes range from mild thickening to marked hypertrophy and hyperplasia, with intimal proliferation and encroachment on the vascular lumen. Fibromuscular hyperplasia commonly develops in the adjacent pulmonary parenchyma and around alveolar ducts. The cat lungworm, *Aelurostrongylus abstrusus*, produces similar lesions, but most cats with medial hypertrophy are parasite-free.

Fibromuscular Dysplasia

Fibromuscular dysplasia (FMD) is an idiopathic, non-inflammatory, non-atherosclerotic segmental vascular wall thickening and central stenotic disease of arteries (and rarely veins).²⁸ Subtype classification is based on the layer of affected vessel wall, i.e. media, intima, or adventitia. Several studies in dogs have demonstrated intramyocardial coronary arterial changes similar to intimal FMD. These lesions are often clinically insignificant, but may develop secondary to pathologic conditions such as subaortic stenosis.⁵ In the author's experience, segmental FMD characterized by stenosis, intimal thickening, and proliferation of SMC in coronary and atrial arteries may be associated with myocardial necrosis, fibrosis, and sudden cardiac death in dogs.

VASCULITIS

A wide variety of agents cause infectious vasculitis, including viruses, bacteria, chlamydia, rickettsia, fungi, protozoa, and helminths. These agents may directly injure ECs or act indirectly through the elaboration of toxins. Non-infectious vasculitides include hypersensitivity and drug reactions, toxins, and uremia. Although the etiology of vasculitis is often unknown, humoral and cell-mediated immunity are thought to play important roles.³ Clinical significance varies depending on the number, size and type of affected vessels, and the presence of thrombosis, ischemia and infarction.

Vasculitis is characterized histologically by the accumulation of leukocytes within and around the vessel wall. Fibrinoid change refers to the deposition of a homogeneous or granular material composed of fibrin,

immunoglobulins, complement, and platelets, with collagen and SMC degeneration. Inflammatory cells may consist primarily of neutrophils, lymphocytes, or macrophages. The presence of fragmented neutrophil nuclei is classified as leukocytoclastic vasculitis, whereas its absence is classified as nonleukocytoclastic vasculitis.

Viral Vasculitis

Equine Herpesvirus-1

Neurotropic strains of equine herpesvirus type 1 (EHV-1) cause an abrupt onset of neurological signs, often due to spinal cord lesions.³³ Grossly, hemorrhagic foci are scattered throughout the brain and spinal cord. Histologic lesions include lymphocytic infiltrates in the media and adventitia, and EC degeneration.

Canine Herpesvirus-1

Canine herpes virus-1 infection has been associated with keratitis, encephalitis, abortion, neonatal mortality, and infertility.²⁴ Serosanguinous pleural and peritoneal effusions are typically accompanied by petechial and ecchymotic hemorrhages scattered throughout the subserosal tissues. Small necrotic foci are found in many organs; however, inflammation and intranuclear inclusions are rare.

Malignant Catarrhal Fever

Malignant catarrhal fever (MCF) is an acute, fulminant disease of cattle, wild ruminants, and occasionally swine. A T-lymphotropic gamma herpes virus causes Wildebeest-associated MCF in exotic hoofstock, whereas ovine herpesvirus 2 causes sheep-associated MCF in cattle. Signs of MCF include generalized lymphoproliferation, vasculitis, and mucocutaneous and cutaneous ulceration. Characteristic vascular lesions consist of focal or segmental perivascular accumulation of herpesvirus infected CD8+ T-cells in many organs.²⁶ Fibrinoid necrosis may affect a single vascular layer or may be transmural. Although mortality rates are high, surviving cattle develop striking proliferative concentric fibrointimal plaques.

Equine Viral Arteritis

Equine viral arteritis (EVA) is caused by a non-arthropod borne enveloped positive stranded RNA virus in the order Nidovirales.⁴ Panvasculitis develops in many organs, causing clinical signs of edema, congestion, hemorrhage, or abortion due to myometritis. Mild vascular changes consist of perivascular edema with EC hypertrophy and lymphocytic infiltrates, whereas severe lesions include fibrinoid necrosis with marked perivascular and vascular lymphocytic and occasional histiocytic infiltrates. Virus localizes to vascular EC, SMC and pericytes, but may be found in a variety of epithelial cell types.

Feline Infectious Peritonitis

Feline infectious peritonitis virus (FIPV) is a single-stranded, positive sense, linear RNA virus composed mainly of nucleocapsid, transmembrane, and spike proteins. FIPV evolves from feline enteric corona virus (FECV) in persistently infected cats in a multistep mutational process. FECV infection generally causes no signs, or mild enteritis. Transition of FECV to FIPV is accompanied by the acquisition of macrophage tropism due to mutations in the C-terminal domain of the spike protein. FIPV infects macrophages and efficiently disseminates when the virus complexes with antibodies against the spike protein, a process referred to as antibody dependent enhancement (ADE). Virus bound to spike protein-specific antibodies is taken up by Fc receptor-mediated endocytosis, with subsequent escape of the viral genome into the cytoplasm.^{25,30} Most FIPV infected cats clear the virus, but fecal shedding may persist for several months. Cats that develop clinical signs are typically less than 2 years of age and mount an ineffective cell-mediated immune response characterized by low CD4+ T-cell counts, decreased levels of IL-12 and IFN- γ , and increased TNF- α and IL-1 β .¹⁷

Effusive (wet) and noneffusive (dry) FIP represent extremes of a clinical continuum. Effusive vasculitis often manifests as abdominal effusion, with serosal surfaces covered by fibrin and perivascular necrosis and inflammation. Pleural and pericardial effusions are less common. Noneffusive FIP affects a variety of organs including eyes, CNS, kidneys, liver, pancreas and intestines. Microscopically, varying proportions of lymphocytes, macrophages, plasma cells and neutrophils accumulate in and around venules. Fibrin deposition correlates with severity of the vasculitis, while vascular degeneration and thrombosis lead to parenchymal necrosis. Noneffusive FIP clinically resembles lymphosarcoma, steatitis, mycotic infection and toxoplasmosis; however, histopathology provides the definitive diagnosis of FIP.

Rocky Mountain Spotted Fever

Rocky Mountain spotted fever is caused by *Rickettsia rickettsii*, a Gram negative bacterium transmitted by regional tick vectors including *Dermacentor variabilis*, *Dermacentor andersonii*, and *Amblyomma americanum*.³¹ *Rickettsia* replicate within ECs of small blood vessels in a wide variety of mammals, but dogs most often develop disease. Clinical signs include myalgia/arthritis, lymphadenopathy, dyspnea, conjunctivitis, edema, vomiting, diarrhea and petechial or hemorrhagic diathesis. Acute meningoencephalitis, often affecting the vestibular system, may be seen as well as inflammation of retinal vessels. Necrotizing vasculitis of small veins, capillaries and arteries is accompanied by predominantly perivascular lymphocytic infiltrates. EC necrosis and host inflammatory response lead to increased vascular permeability, edema, and multifocal hemorrhage and thrombosis.

Verminous Arteritis

Strongylus Vulgaris

Strongylus vulgaris is the most serious of the large and small strongyles that infect horses.⁷ Larvae are unable to penetrate the internal elastic lamina and therefore migrate within the vascular intima. After reaching the cranial mesenteric artery, larvae undergo 3–4 months of development before molting and returning to the wall of the cecum and colon. Tortuous intimal tracks, consisting of fibrin, necrotic cellular debris, and mixed inflammatory cells are eventually covered by ECs and converted into fibroelastic thickenings. Clinical signs develop secondary to occlusive thrombi, often in the cranial mesenteric artery but also found in the aorta, renal arteries, and celiac artery. Affected vessels may develop saccular or fusiform aneurysms due to weakening of the wall. Cecal and colic artery thromboembolism, pressure on abdominal autonomic plexuses, and release of toxins from dead larvae induce colic.

Heartworm Disease

Heartworm disease caused by *Dirofilaria immitis* is common in dogs, but occurs in other mammals including domestic and wild felids, wild canids, ferrets, sea lions, muskrats, and horses. Mosquitoes of the genera *Aedes*, *Culex*, and *Anopheles* transmit infective third stage larvae that migrate through connective tissues to veins, ultimately reaching the heart 3–4 months later where they mature to adults. Adult worms reside primarily in the pulmonary arteries and right ventricle, but may extend into the right atrium and vena cava in heavy infestations. Pulmonary veins in the caudal lung lobes often develop fibromuscular intimal proliferation, resulting in pulmonary hypertension and right heart failure. Worm larvae, adults, and an intracellular bacteria *Wohlbachia*, induce eosinophilic and neutrophilic endoarteritis, and rugose myointimal proliferation in the host. Antidote therapy may exacerbate thromboembolism, pulmonary hypertension, and induce granulomatous reactions.

Photosensitization Dermatitis

Photosensitization dermatitis occurs in animals that ingest plants containing photodynamic agents or drugs (type I), have an inherent inability to metabolize heme pigments (type II) or have liver disease that impairs excretion of phylloerythrin, a degradation product of chlorophyll (type III). Type III or hepatogenous photosensitization is associated primarily with cholestatic liver disease. Gross lesions occur in lightly colored areas of skin exposed to sunlight, with histopathologic findings of fibrinoid vascular degeneration, dermal edema, and epidermal coagulation necrosis.¹⁴

Immune-mediated Vasculitis

Among the various immune mechanisms causing vasculitis, antigen-antibody complexes formed in the cir-

ulation (type II hypersensitivity) or locally (type III hypersensitivity) are most common. Complexes precipitate in vascular walls and bind to neutrophils via Fc or C3b receptors, thereby triggering inflammation, proinflammatory cytokine and lysosomal enzyme release, and generation of oxygen-derived free radicals. Complement cascade activation leads to neutrophil and monocyte recruitment and increased vascular permeability. Immune complex injury results in vascular wall necrosis and fibrinoid changes. Lesions may resolve as immune complexes are catabolized.

Equine Purpura Hemorrhagica

Equine purpura hemorrhagica occurs in 1–2% of horses after acute infection with *Streptococcus equi*.²⁰ Deposits of streptococcal M protein complexed with IgA or IgM damage ECs, and induce systemic leukocytoclastic vasculitis. Head and hind leg edema is accompanied by petechia on mucosal and serosal surfaces. Serious sequelae include immune complex glomerulonephritis and infarction of skeletal muscle, skin, gastrointestinal tract, pancreas and lungs, similar to the human syndrome Henoch-Schönlein purpura.

Canine Juvenile Polyarteritis Syndrome

Canine juvenile polyarteritis syndrome (CJPS) occurs primarily in young Beagles, but has been documented in a variety of breeds.^{6,29} Affected dogs have sudden onset fever, anorexia, and a hunched stance, with frequent remissions and relapse. Small to medium muscular arteries in the dura mater and leptomeninges of the cervical spinal cord, extramural coronary arteries, and cranial mediastinum develop segmental vasculitis and perivasculitis. Lymphocytes, macrophages, and plasma cells encircle the vessel and often expand into an eccentric nodule, with neutrophils predominating in acute lesions. Fibrinoid change and rupture of the elastic lamina, and fibrin thrombi, hemorrhage, and necrosis of adjacent tissues characterize severe lesions. CJPS has many features of an immune complex syndrome of children, Kawasaki disease, including high serum IgA, low peripheral T-cells and high B-cells, mononuclear activation, antineutrophil cytoplasmic antibodies, and suppressed mitogenic response. Other causes of canine systemic vasculitis include leishmaniasis and drug toxicities.

Paraneoplastic Vasculitis

Paraneoplastic leukocytoclastic vasculitis with variable degrees of necrosis, is occasionally seen in association with lymphosarcoma.¹⁸

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Treatment of Hemostatic Defects

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Treatment of Hemorrhagic Disorders

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Acronyms and Abbreviations

ACT, activated clotting time; AT, antithrombin; aPTT, activated partial thromboplastin time; BSA, bovine serum albumen; CE, cardiogenic embolism; CNS, central nervous system; COX, cyclo oxygenase; DDAVP, desamino-8-D-arginine vasopressin (desmopressin); DIC, disseminated intravascular coagulation; EACA, ϵ -aminocaproic acid; Hct, hematocrit; HIT, heparin-induced thrombocytopenia; IM, intramuscular; IMHA, immune-mediated hemolytic anemia; IMT, immune-mediated thrombocytopenia; INR, international normalized ratio; IV, intravenous; LMWH, low molecular weight heparin; PIVKA, proteins induced by vitamin K absence/antagonism; PT, prothrombin time; PTE, pulmonary thromboembolism; SC, subcutaneous; TTP, thrombotic thrombocytopenic purpura; UH, unfractionated heparin; uPA, urokinase; VWD, von Willebrand disease; VWF, von Willebrand factor.

Hemostatic defects, arising from either inadequate or uncontrolled hemostatic plug formation, cause significant mortality if unrecognized or inappropriately treated. The hemorrhagic disorders discussed in this section include platelet defects (thrombocytopenia and platelet dysfunction) and plasma protein defects (coagulation and von Willebrand factor [VWF] deficiencies). Management of thrombosis arises in the context of primary immune, endocrine, cardiac, and neoplastic disorders. Although empiric use of blood components and antithrombotic agents is common in veterinary practice, evidence-based guidelines for defined disease syndromes have not yet been developed.

TREATMENT OF HEMORRHAGIC DISORDERS

Transfusion Therapy

Patients with hemorrhagic disorders are often transfused to control signs of active bleeding, prevent abnormal bleeding from invasive procedures, or correct signs of blood loss anemia. Blood component transfusion is usually the best means to provide sufficient cells or proteins to support hemostatic plug formation, while minimizing risk of volume overload and sensitization to foreign antigens²⁵ (see Section VIII). The blood components used to treat hemorrhagic disorders include

TABLE 90.1 Blood Products for Hemorrhagic Disorders

Products	Dosage Guidelines	Indications
Platelet rich plasma ^a	6–10 mL/kg	Thrombocytopenia, platelet dysfunction
Fresh frozen plasma	6–12 mL/kg	Coagulation factor and VWF deficiencies
Cryoprecipitate ^b	1 unit/10 kg	Fibrinogen, factor VIII, and VWF deficiencies
Cryo-poor plasma	6–12 mL/kg	Deficiencies of factors II, VII, IX, X, and XI
Fresh whole blood	12–20 mL/kg	Replacement of platelets and all factors

^aPlatelet rich plasma containing at least 200×10^6 platelets/mL.

^b1 unit cryoprecipitate prepared from 200 mL fresh frozen plasma.

platelet rich plasma and cell-free plasma products (see Table 90.1).

Transfusion may transiently correct underlying deficiencies; therefore baseline samples (EDTA and citrate blood) for laboratory assays and point-of-care tests should be collected before transfusion. At minimum, platelet count and coagulation screening tests (e.g. activated clotting time [ACT], activated partial thromboplastin time [aPTT], prothrombin time [PT]) should be evaluated in any patient suspected of having a hemorrhagic disorder. Sophisticated viscoelastic coagulation analyzers are validated to guide transfusion for human patients undergoing liver transplantation and cardiac bypass.¹⁴ Among these instruments, the thromboelastograph is most actively under investigation for veterinary patients.^{7,49} In vitro assays, however, cannot replace careful patient monitoring. Cessation of hemorrhage and stabilization of hematocrit (Hct) are defined clinical endpoints for confirming appropriate response.^{18,24}

Transfusion for Platelet Defects

Platelet defects include thrombocytopenia, and acquired and hereditary platelet dysfunction (see Section VI). Medical therapy is the primary means of managing most of these disorders. Platelet transfusion is rarely beneficial for immune-mediated thrombocytopenia (IMT) because transfused platelets are removed rapidly from circulation. Acquired platelet function defects typically cause mild to moderate bleeding tendencies that resolve after correction of the underlying disorder. Most patients with hereditary platelet dysfunction have sporadic bleeds, and require transfusion primarily for surgical prophylaxis (see Chapter 97). The goal of platelet transfusion is replacement of sufficient functional platelets to support hemostasis, rather than normalization of platelet count. In human medicine, threshold platelet counts as low as 5,000–10,000/ μ L are considered safe for patients with chronic thrombocytopenia, whereas counts of 50,000/ μ L up to 100,000/ μ L are used for active hemorrhage, including massive trauma and CNS injury.¹⁸

Transfusion for Coagulation and von Willebrand Factor Defects

Simultaneous deficiency of many coagulation factors develops in patients with severe liver disease, vitamin

K deficiency, overt hemorrhagic disseminated intravascular coagulation (DIC), and massive transfusion (see Chapter 85). In contrast, most hereditary defects involve deficiency of a single factor (see Chapters 81 and 86). Unlike platelet defects, transfusion therapy is the primary means for controlling or preventing hemorrhage in patients with acquired and hereditary factor deficiencies. Plasma components, rather than whole blood, provide rapid replacement of hemostatic factors, while minimizing risks of sensitization and volume overload (Table 90.1 and Chapter 96). Measurement of in vitro clotting times and the use of newer point-of-care analyzers can aid in patient monitoring; however, clinical status is the most important parameter for determining adequate response to plasma (and platelet) transfusion. Clinical outcome variables to gauge response include the severity, site, and duration of hemorrhage, change in Hct, and transfusion intervals.^{18,24}

Non-Transfusion Therapy

Drug Therapy

Pharmacologic agents may reduce or replace transfusion for some patients with hemorrhagic disorders. Non-transfusion therapy eliminates the risk of disease transmission and transfusion reaction, and promotes more efficient use of scarce blood supplies. Most drug therapies, with the exception of vitamin K, indirectly support hemostasis rather than reverse a primary hemorrhagic condition (see Table 90.2).

Vitamin K Therapy Vitamin K deficiency is a common acquired coagulopathy, often arising due to anticoagulant rodenticide toxicity or liver disease (see Chapter 85). Vitamin K replacement should be given in the form of vitamin K₁ (phytonadione), rather than vitamin K₃ (menadione). Oral administration is appropriate for patients with no gastrointestinal signs or malabsorptive disorders. The subcutaneous (SC) route is preferred for parenteral vitamin K₁, because IV administration may cause anaphylaxis, and intramuscular (IM) injection risks hematoma formation. Vitamin K₁ at an initial dose of 2–4 mg/kg, with maintenance dosage of 1.5 mg/kg q12–24 hours provides adequate replacement therapy, without risk of inducing Heinz body formation.¹³ Factor synthesis to correct coagulopathy occurs over a lag

TABLE 90.2 Drug Therapy for Hemorrhagic Disorders

Drugs	Dosage Guidelines	Indications
Vitamin K ₁	2 mg/kg/day PO ^d or SQ	Vitamin K-dependent coagulopathy
Desmopressin	1 μg/kg SQ ^e	Type 1 VWD, platelet dysfunction
EACA ^a	100 mg/kg PO or IV (loading); 30 mg/kg/h	Perioperative hemorrhage
Tranexamic acid ^b	10 mg/kg IV; 25 mg/kg PO q8 h	Perioperative hemorrhage
rhVIIa ^c	35–120 μg/kg IV	Coagulation inhibitors, refractory hemorrhage, surgical hemostasis

^aLoading dose given as single bolus followed by hourly dose for up to 8 h.

^bMaintain for up to 7 days post-operatively.

^cDoses given q2 h until control of hemorrhage.

^dOrally

^eSubcutaneously

period of up to 12–24 hours from initiation of therapy. Transfusion is indicated, therefore, to immediately control hemorrhage in patients with signs of respiratory tract or CNS hemorrhage, or severe blood loss anemia. Prolonged vitamin K₁ therapy (up to 6 weeks) may be required to maintain hemostasis for long-acting anticoagulant rodenticide toxicities. Samples for coagulation testing should be drawn pre-treatment, because diagnostic laboratory findings of vitamin K deficiency (e.g. prolonged aPTT, PT, proteins induced by vitamin K absence/antagonism [PIVKA]) are corrected concomitantly with clinical reversal of the bleeding tendency (see Chapter 138).

Desmopressin Desmopressin (DDAVP) is a synthetic analogue of arginine vasopressin developed to produce antidiuresis without vasoactive side-effects.²⁷ Desmopressin promotes hemostasis by increasing plasma VWF concentration via release of intracellular stores. The drug also enhances platelet function via less-defined, non VWF-dependent mechanisms. It is most often used in human medicine to treat patients with mild to moderate forms of VWD and hemophilia A; however, it has shown some benefit for patients with hereditary and acquired platelet function defects.²⁷ The action of desmopressin varies among species. Dogs demonstrate relatively low qualitative and quantitative VWF release; nevertheless, desmopressin has been reported to shorten in vivo bleeding time and improve surgical hemostasis in dogs with type 1 VWD and platelet dysfunction.⁹ The empiric dosage for this use is 1 μg/kg SC. The maximal increment in plasma VWF occurs by 1 hour post administration, with no additional benefit expected from repeated doses. Response varies among individuals; therefore blood products should be available if drug therapy fails to control hemostasis.

Antifibrinolytic Drugs The lysine analogues, ε-aminocaproic acid (EACA) and tranexamic acid, delay fibrinolysis by impairing plasminogen's interaction with fibrin. Both drugs are given systemically to reduce transfusion requirements for people undergoing surgery associated with large volume blood loss (e.g. liver transplantation, joint replacement, cardiac bypass; Table 90.2).²⁶ In addition, tranexamic acid is used as a topical solution for patients with hereditary and acquired coagulopathies undergoing oral surgery. Aprotinin, a serine protease inhibitor of plasminogen, has also proven effective for reducing perioperative blood loss and transfusion requirements in people; however, recent studies suggest that aprotinin therapy is associated with renal failure. The pharmacokinetics of EACA in horses has recently been described, and this drug has been used empirically in horses to control hemorrhage secondary to traumatic, surgical, and infiltrative disease.³⁸ The lysine analogues have few serious side-effects in people and their clinical use in veterinary medicine warrants further investigation.

Recombinant FVIIa Supraphysiologic levels of activated FVII (FVIIa) are capable of bypassing the role of factors VIII and IX in promoting thrombin formation. Recombinant FVIIa (rhFVIIa) was initially developed to treat hemorrhagic episodes in hemophiliacs with coagulation inhibitors. In addition to this approved use, rhFVIIa is undergoing trials for controlling hemorrhage in a variety of surgical procedures (e.g. spinal surgery, liver transplantation) and for severe intracranial and post-traumatic bleeds.³⁷ The approved dosage regimen involves intravenous bolus (90 μg/kg) at 2 hour intervals until cessation of bleeding (Table 90.2). While rhFVIIa appears safe and effective in hemophiliacs, its wider use has been associated with thrombotic complications. Efficacy studies have been performed in hemophilic and factor VII deficient dogs; however, the drug's expense and risk of sensitization will likely limit clinical veterinary use.

Topical Agents

A variety of topical agents, classified as hemostats, sealants, and adhesives, have been developed for direct application to control bleeding and/or close tissue surfaces at sites of surgical or traumatic injury (Table 90.3).⁴²

Hemostats provide a mechanical barrier and matrix for fibrin clot formation and include products derived from porcine gelatin, bovine collagen, cellulose, and polysaccharide spheres. Thrombin, the enzyme that directly transforms fibrinogen to fibrin, is used in combination with hemostats to enhance their activity and in a two-component plasma-derived fibrinogen product, fibrin sealant. Synthetic products include cyanoacrylate adhesives for superficial skin closures and polyethylene glycol sealants approved for durotomy and abdominal procedures. A two-component adhesive consisting of bovine serum albumen (BSA) and glutaraldehyde is also approved for cardiac and vascular surgery. The

TABLE 90.3 Topical Hemostatic Agents

Category	Products (Tradename, Source)
Hemostats: Mechanical	Porcine gelatin (Gelfoam, Pharmacia) Bovine collagen (Avitine, Bard; Helistat, Integra; Instat, Johnson & Johnson) Cellulosic (Surgicel, Johnson & Johnson) Polysaccharide spheres (Arista, Medafor)
Hemostats: Flowable	Human thrombin with bovine gelatin (Flo seal, Baxter) Porcine gelatin matrix (Surgiflo, Johnson & Johnson)
Thrombin products	Bovine thrombin (Thrombin-JMI, King Pharmaceuticals) Human-derived (Evithrom, Johnson & Johnson) Recombinant human (Recothrom, Zymogenetics)
Fibrin sealants	Human plasma derived fibrin plus thrombin (Tisseal, Baxter; Evicel, Johnson & Johnson)
Synthetic sealants	Polyethylene glycol polymers (Coseal, Baxter; Duraseal, Covidien)
Adhesives	Cyanoacrylate (Dermabond, Johnson & Johnson; Indermil, Covidien; Histoacryl, Tissueseal) Glutaraldehyde and albumen (Bioglue, Cryolife)

potential side-effects of the topical agents include allergic reactions, sensitization to heterologous proteins, embolization, wound infection, and delayed healing.

TREATMENT OF THROMBOTIC DISORDERS

Pathologic thrombosis reflects an imbalance between fibrin formation and fibrinolysis, arising from anticoagulant or fibrinolytic pathway dysfunction, and/or an overwhelming excess of procoagulant stimuli (see Chapters 87 and 88). Numerous hereditary thrombotic syndromes or “thrombophilias” have been identified in people. Among the best characterized are factor V_{Leiden} (a defect that renders factor V insensitive to protein C inactivation) and deficiencies of the anticoagulant proteins, antithrombin (AT) and protein C. To date, hereditary thrombophilias have not been identified in domestic animals.

Clinical Thrombotic Disorders

Thrombosis is recognized as a common complication of many common diseases in animals. Observational studies include immune-mediated hemolytic anemia (IMHA), hyperadrenocorticism, cardiac disease, and neoplastic and protein losing disorders as primary disease conditions often associated with systemic thrombosis and pulmonary thromboembolism (PTE) (see Chapter 87). Although hemostatic abnormalities

are often described, the specific causal factors of thrombosis in animals with these conditions remain ill-defined. Regardless of underlying mechanisms, thrombosis increases morbidity and mortality beyond that of the primary disease and often prompts consideration of antithrombotic therapy.

Antiplatelet Drugs

Aspirin

Aspirin is the most used and studied antiplatelet agent.¹² It is an indirect agent that inhibits secondary platelet aggregation by irreversibly acetylating platelet cyclooxygenase (COX), thereby preventing the formation of the platelet agonist, thromboxane A₂. Aspirin also inhibits endothelial cell COX activity, resulting in decreased prostacyclin synthesis and loss of anti-aggregating and vasodilating properties. Unlike platelets, endothelial cell COX inhibition is reversible and therefore antithrombotic conditions predominate at sites of intact endothelium.

The prophylactic benefit of aspirin on arterial thrombosis in people is confirmed by large meta-analyses demonstrating reduced death, myocardial infarction, and stroke due to coronary syndromes.² Aspirin is relatively ineffective, however, in primary or secondary prevention of cardiogenic embolism (CE) in patients with prothrombotic conditions such as atrial fibrillation.

The pharmacologic, analgesic, and antiplatelet effects of aspirin have been well studied in dogs and cats.^{5,7} Aspirin has been used in cats for over 30 years with the goal of preventing CE; however, retrospective studies reveal recurrence rates of CE from 17% to 75%.^{31,34,39} Adverse gastrointestinal effects (e.g. anorexia and vomiting) are common in aspirin-treated cats, but can be ameliorated by low-dose therapy (5 mg/cat q72 hours).³⁹ Although few clinical reports describe aspirin anti-thrombotic therapy in dogs, a retrospective study of IMHA attributed improved clinical outcomes to the addition of low-dose aspirin to immunosuppressive therapy.⁴⁶ Healthy dogs receiving aspirin uniformly develop gastroduodenal erosions and submucosal hemorrhages; however, clinical signs are uncommon.³⁶

Thienopyridines

Thienopyridines induce specific and irreversible antagonism of the platelet membrane ADP_{P2Y12} receptor.^{3,28} These agents are more potent than aspirin and are classified as direct antiplatelet agents, inhibiting both primary and secondary platelet aggregation in response to multiple agonists. The parent compounds must undergo hepatic biotransformation to form active metabolites. Inhibition of the ADP_{P2Y12} receptor by the active compounds decreases platelet release of serotonin, ADP and thromboxane, thereby inhibiting the ADP-induced conformational change of platelet glycoprotein IIb/IIIa and reducing fibrinogen and VWF binding. Modulation of platelet-mediated vasoconstriction also occurs.²⁸

Ticlopidine Ticlopidine (Ticlid®) is a first generation thienopyridine with a delayed, 4–6 day, onset of action. In spite of its demonstrated efficacy in people, its use is limited by adverse events such as gastrointestinal and dermatologic disturbances, bleeding, agranulocytosis, altered hepatic tests and thrombotic thrombocytopenic purpura (TTP).²⁸

A pharmacodynamic study in normal cats demonstrated consistent antiplatelet effects of high-dose ticlopidine; however, gastrointestinal side-effects precluded its clinical use.²¹ Similarly, gastrointestinal side-effects were found in heartworm infested dogs treated with ticlopidine.^{6a}

Clopidogrel Clopidogrel (Plavix®) is a second-generation thienopyridine that has supplanted ticlopidine because of its equal or better clinical efficacy, and more favorable safety profile.¹¹ Clopidogrel's maximal antiplatelet effects are achieved by 3–5 days of daily administration and its action wanes by 5–7 days after discontinuation. Adverse effects of clopidogrel in people include diarrhea, skin rash, occasional minor bleeding, and a possible risk of TTP. Large clinical trials demonstrate that clopidogrel significantly reduces the risk of stroke, myocardial infarction and vascular death compared to aspirin.¹⁰

In a pharmacodynamic study in normal cats, clopidogrel induced a 95% inhibition in platelet aggregation in response to ADP, 92% inhibition in serotonin release and a 3.9-fold prolongation in mucosal bleeding time.²² While no adverse effects were noted in this study, anecdotal reports describe vomiting in some cats that responds to administration of the drug in a gel capsule or with food. To date, there is no evidence of TTP in clopidogrel treated-cats. Clopidogrel administration improved collateral circulation and neurologic function in a feline model of aortic infarction.²³ A randomized controlled trial now in progress, the Feline Arterial Thromboembolism: Clopidogrel vs Aspirin Trial (FAT CAT; <http://www.vin.com/fatcat/>) is designed to evaluate the drug's clinical efficacy for feline CE. The pharmacodynamics of clopidogrel in dogs has been investigated, but clinical treatment trials have not been performed.¹⁶

Anticoagulant Drugs

Unfractionated Heparin

Unfractionated heparin (UH) is derived from porcine intestine or bovine lung and consists of heterogeneous molecules with a mean molecular weight of approximately 15,000 Da (range 3,000–30,000 Da).²⁰ This heterogeneity produces highly variable pharmacokinetic and anticoagulant properties. Heparin molecules contain a pentasaccharide sequence that binds to AT, thereby facilitating AT's inhibition of factors IIa (thrombin), Xa, and IXa, and reducing thrombin-catalyzed activation of factors V and VIII. The most common adverse effect of UH therapy is bleeding; however, a severe thrombotic syndrome of heparin-induced thrombocy-

topenia (HIT) develops in up to 10% of UH-treated people. UH has proven efficacy in people for prevention or treatment of venous thrombosis, PTE, acute coronary syndrome, cardiac surgery, and cardiovascular interventions.²⁰

Unfractionated heparin has been used for many veterinary conditions including DIC, IMHA, PTE, and arterial thromboembolism. Treatment regimens are generally empiric, or extrapolated from the human literature. Because of its highly variable pharmacokinetics, UH therapy requires individual patient monitoring. The aPTT is typically used to monitor UH, with a therapeutic goal of prolongation to 1.5–2 times baseline values. In addition to the aPTT, UH monitoring can be based on measurement of factor Xa inhibitory activity (anti-Xa assays).²⁰

Low Molecular Weight Heparins

The low molecular weight heparins (LMWHs) are smaller in size than UH (4,000–5,000 Da), but maintain the pentasaccharide sequence that binds to AT.^{4,20} LMWHs inhibit factor IIa to a much lesser degree than factor Xa. Due to their smaller size, the LMWHs also have a higher bioavailability and longer plasma half-life than UH. At therapeutic levels, the LMWHs do not prolong aPTT; therefore anti-Xa assays are used for monitoring clinical studies.⁴⁵ Peak anti-Xa levels in people occur at 3–5 hours after SC injection, with decline to undetectable levels by 8 hours. Although anti-Xa activity does not directly correlate with in vivo anticoagulant effect, positive clinical outcomes have been associated with peak LMWH anti-Xa activities of 0.5–1.0 U/mL.²⁰ For clinical use, LMWHs are typically administered at a fixed dose with no laboratory monitoring. Numerous human trials have demonstrated that LMWH produce equivalent or improved outcomes versus UH for unstable angina, myocardial infarction, venous thrombosis, and PTE.²⁰ Bleeding is the most common adverse effect of LMWH, with reported frequencies of up to 27% (minor bleeding) and up to 6% (major bleeding).

The LMWHs have generated considerable interest in veterinary medicine. Pharmacokinetic studies of dalteparin (100 U/kg) and enoxaparin (1 mg/kg) given SC to healthy cats demonstrate attainment of peak anti-Xa activities by 4 hours, with decline to undetectable levels by 8 hours.¹ In a pharmacodynamic study in cats using a venous stasis model, enoxaparin (1 mg/kg q12 hours) induced 91.4% thrombus inhibition at 12 hours after drug administration, in the absence of measurable anti-Xa activity.⁴⁴ This dosage regimen may provide therapeutic anticoagulant levels; however, clinical trials are needed to confirm its efficacy. Retrospective studies of variable dosage dalteparin therapy in cats for secondary prevention of CE revealed comparable survival times to warfarin-treated cats, and relatively few side-effects.⁴⁰ The pharmacokinetics of enoxaparin and dalteparin have also been investigated in healthy dogs; however, clinical trials have not yet defined effective dosage regimens.²⁹

Warfarin

Warfarin blocks vitamin K recycling which results in production of dysfunctional vitamin K-dependent coagulation factors (factors II, VII, IX and X), and anticoagulant proteins C and S. Warfarin remains the standard oral drug for thromboprophylaxis of various human conditions including venous thrombosis, atrial fibrillation, and prosthetic heart valves.³³ Bleeding is the most common complication, with major bleeding rates of approximately 1–2%, and up to 20% for minor bleeding. Warfarin metabolism is influenced by genetic factors and drug interactions that may increase or decrease its anticoagulant effect. Warfarin therapy is adjusted by transformation of PT assay results to international normalized ratio (INR) values. The INR allows standardization among testing laboratories and is derived from the following equation: $[(\text{patient PT}/\text{control PT})^{\text{ISI}}]$, where ISI is a measure of PT reagent sensitivity.³⁰ Although clinical trials based on dosage to target INR have not been performed in animals, INR values of 2–3 are associated with effective thromboprophylaxis in people and are recommended for most thrombotic syndromes.³³

The pharmacokinetics and pharmacodynamics of warfarin have been evaluated in dogs and cats.^{32,41} The human guidelines of PT prolongation to 1.3–1.6 times baseline, or an INR of 2–3 are empiric therapeutic targets for anticoagulation for animals. Retrospective studies of warfarin for secondary prevention of CE in cats reveal recurrence rates of 40–50%, with hemorrhage (including fatal hemorrhage) the most common complication.³¹ Owners must be advised of the risks, expense, and dedication needed to undertake warfarin therapy in animals.

Synthetic Xa Inhibitors

Synthetic Xa inhibitors are a new class of drugs designed to selectively inhibit factor Xa through potentiation of AT.⁴ These agents are homogeneous preparations of pentasaccharide units that bind exclusively to AT. These drugs also have complete bioavailability when administered subcutaneously, demonstrate less variability, and do not cause HIT. Drug monitoring is based on measurement of FXa inhibition in anti-Xa activity assays. The most common adverse effect is bleeding, occurring with a similar frequency as LMWH.

Fondaparinux

Fondaparinux (Arixtra®) is currently approved for medical use. The drug potentiates AT's anti-Xa activity by approximately 300-fold, has an elimination half-life of 17–21 hours and is given at 24 hour intervals.¹⁵ Careful monitoring is required in patients with renal insufficiency because it is excreted unchanged in the urine. Numerous clinical trials have demonstrated fondaparinux equivalence or superiority to the LMWH, enoxaparin, for thromboprophylaxis in patients under-

going hip or knee replacement surgery, or for treatment of deep venous thrombosis or PTE.⁸ A pharmacokinetic study of fondaparinux in cats demonstrated that dosage of 0.06 mg/kg SC q. 12h results in peak and trough anti-Xa values that approximate human therapeutic levels (Hogan DF, unpublished data).

Idraparinux

Idraparinux is a second-generation synthetic pentasaccharide designed to replace warfarin for long-term thromboprophylaxis. Idraparinux binds to AT with high affinity, resulting in a plasma half-life equivalent to that of AT (80 hours).¹⁹ Preliminary studies indicate that idraparinux is generally as effective as warfarin for patients with venous thrombosis and atrial fibrillation; however, its use is associated with higher rates of abnormal bleeding. The increased risk of bleeding and lack of specific antidote have slowed further development of idraparinux for clinical use.

Fibrinolytic Drugs

Supraphysiologic levels of plasminogen activators are administered intravenously to cause clot dissolution in patients with venous thrombosis, PTE, systemic arterial occlusive disease, ischemic stroke and acute myocardial infarction.^{17,43} The most common complication of thrombolytic therapy in people is bleeding, which may be caused by thrombocytopenia, platelet dysfunction, hypofibrinogenemia, systemic fibrinolysis, or clot disruption. Reperfusion injury is another complication seen when metabolic waste and electrolytes are released from infarcted tissues. Leriche's syndrome refers to ischemia of the pelvic limb musculature caused by distal aortic infarction. Thrombolytic therapy in these patients often induces severe metabolic acidosis and hyperkalemia. An analogous condition develops in cats with aortic CE post-thrombolytic therapy.³⁴ Of the many thrombolytic agents approved for use in people, veterinary reports are limited to t-PA, urokinase, and streptokinase.

Tissue Plasminogen Activator

Plasminogen and tissue plasminogen activator (t-PA) both have a high affinity for fibrin, and t-PA interacts preferentially with fibrin-bound rather than free plasma plasminogen.⁴³ However, t-PA's fibrin specificity is relative and at high doses it induces a systemic proteolytic state and bleeding.¹⁷ Isolated reports of dogs with arterial thromboembolism or vena caval thrombosis treated with bolus human recombinant t-PA (Activase®) describe variable response and hemorrhagic complications.⁶ A clinical trial of t-PA therapy in six cats with CE revealed an acute survival rate of 50%, with deaths attributable to reperfusion injury and cardiogenic shock.³⁴ The survivors had perfusion restored within 36 hours and return to motor function within 48 hours.

Streptokinase

Streptokinase is considered a “nonspecific” plasminogen activator because it converts both circulating and fibrin-bound plasminogen to the active enzyme, plasmin.⁴³ Systemic plasminemia may predispose to bleeding from degradation and loss of fibrinogen and coagulation factors, and the inhibitory effects of fibrin breakdown products. Streptokinase is an immunogenic protein that can elicit allergic reactions and reduced drug efficacy, especially upon repeated administration. Streptokinase is rarely given to people at less than 4 year intervals. Streptokinase (Streptase®) has been administered to dogs and cats as an intravenous infusion of 90,000 IU intravenously (IV) over 1 hour followed by 45,000 IU/hour for up to 12 hours. Severe complications were seen in two retrospective studies of streptokinase therapy in cats with CE and aortic infarction, including acute respiratory distress, hemorrhage, reperfusion injury, and mortality rates of 100% and 67%, respectively.³¹ In contrast, streptokinase therapy for thromboembolic disease is apparently well-tolerated in dogs. In a case series of four dogs, three experienced complete thrombus resolution and all had partial or complete resolution of signs with only rare, minor bleeding.³⁵

Urokinase

Urokinase (uPA) is a serine protease plasminogen activator. Its active, low molecular mass form binds with great affinity to the lysine-plasminogen form of plasminogen, which preferentially accumulates within thrombi. This property confers some fibrin-specificity to uPA. Urokinase (Abbokinase®) is used clinically as a thrombolytic agent in people with venous thrombosis, PTE, and cardiac syndromes.¹⁷ The drug has been administered to cats and dogs with arterial thromboembolism with a protocol of 4,400 IU/kg loading dose given over 10 minutes followed by 4,400 IU/kg/h for 12 hours.^{47,48} In a small case series of cats with CE, uPA was generally well-tolerated and approximately half the cats regained some motor function and ultimately survived.⁴⁷ In contrast, none of four dogs treated with uPA survived.⁴⁸

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Overview of Avian Hemostasis

KENDAL E. HARR

Comparative Avian Hemostasis

- Tissue Factor and Active Factor Complexes
- Contact Factor Pathway
- Fibrinogen Formation and Fibrinolysis
- Thrombocytes

Sample Collection for Coagulation Testing

- Venipuncture Technique
- Blood Collection and Processing

Coagulation Testing

- Prothrombin Time
- Activated Clotting Time
- Fibrinogen

Avian Hemorrhagic Disorders

- Vitamin K Deficiency
- Hepatotoxins and Hepatic Disease
- Infectious Disease
- Treatment Guidelines

Acronyms and Abbreviations

ACT, activated clotting time; aPTT, activated partial thromboplastin time; DIC, disseminated intravascular coagulation; HPAI, highly pathogenic avian influenza; PK, prekallikrein; PT, prothrombin time.

Coagulation is a highly conserved vertebrate defense mechanism for controlling hemorrhage. Phylogenetic and comparative sequence analyses reveal that the key mammalian coagulation proteases (factors II, VII, IX, X) and cofactors (factors V and VIII) are present in birds and evolved from common ancestral genes.⁴ However, differences between birds and mammals in coagulation protein structure and function affect the clinical assessment of avian hemostasis. Assay methods and reagents optimized for mammals are not directly applicable for the diagnosis of avian coagulopathies.^{1,5,7,28} Although hemorrhagic disorders are recognized in poultry, pet birds, and wildlife, their clinical diagnosis is challenging due to relative unavailability of valid assay techniques.

COMPARATIVE AVIAN HEMOSTASIS

Coagulation in birds, as in mammals, culminates when a burst of thrombin (factor IIa) transforms soluble plasma fibrinogen into an insoluble fibrin clot.⁹ Coagulation is initiated by tissue factor/factor VIIa and then amplified and propagated by factor complexes (factor VIIIa/IXa and factor Va/Xa). The major inhibitors of

coagulation, Protein C and antithrombin, are also present in birds (see Chapter 84).^{4,6}

Tissue Factor and Active Factor Complexes

In response to injury, the tissue factor/factor VIIa complex activates factors IX and X. These serine protease factors assemble on cell membrane phospholipids with their respective cofactors (factors VIII and V) to form active enzyme complexes. The corresponding avian factor and cofactor genes share extensive sequence homology with their mammalian counterparts.⁴ For example, functional regions of ostrich prothrombin demonstrate 70–87% identity with the bovine and human genes. The encoded avian protein retains the overall domain structure of mammalian prothrombin and consists of an open reading frame of 607 amino acids compared to 608 amino acids for human prothrombin.⁸ In addition, the enzymatic activities of human and avian thrombin and factor Xa are similar based on their cleavage of small peptide substrates.⁹ Avian factors have relatively low activities, however, in clotting time assays configured with mammalian plasma and tissue thromboplastins.^{4,9,28} In spite of functional similarities, avian and mammalian coagulation factors

have structural differences that influence their ability to form active enzyme complexes in heterologous (cross-species) systems.

Contact Factor Pathway

The contact pathway, found in most mammals, includes three serine protease factors: factor XI, factor XII, and prekallikrein (PK). These factors are required for surface-initiated *in vitro* fibrin formation in the activated partial thromboplastin time (aPTT) screening test. *In vivo* deficiencies of these factors, however, cause either mild (factor XI) or no signs of a bleeding diathesis. Comparative genomic studies have recently described their evolutionary development and clarified the cause of prolonged contact-activated clotting times of avian plasma.¹⁹ These analyses indicate that a single prekallikrein-factor XI “predecessor” gene was retained in birds and did not undergo the duplication event leading to two distinct mammalian genes. In contrast, an ancestral vertebrate factor XII gene was lost from the lineage leading to birds. Consequently, aPTT screening tests and factor assays optimized for activation of the mammalian contact pathway are not directly applicable for assessing avian coagulation.^{5,19} Further studies are needed to develop a comparable aPTT screening test method for birds.

Fibrinogen Formation and Fibrinolysis

Fibrinogen in birds, as in mammals, functions as a hemostatic protein and a positive acute phase reactant.^{11,12} Avian plasma fibrinogen concentration is approximately 100–300 mg/dL (1–3 g/L).¹¹ The fibrinogen molecule in all species consists of three polypeptide chains. Although certain structural features differ between species, avian fibrinogen is susceptible to cleavage by mammalian thrombin. Factor XIII is also conserved in birds and acts to cross-link mature fibrin. Fibrinolysis is not fully characterized in birds; however, avian homologs have been identified for the fibrinolytic pathway serine proteases (i.e. urokinase, tissue plasminogen activator, and plasminogen).¹⁹

Thrombocytes

Thrombocytes in non-mammalian vertebrates fulfill the function of platelets as cellular matrices of the hemostatic plug, and have additional phagocytic activities not displayed by platelets. Thrombocyte counts of normal birds typically range from 20,000–30,000/ μ L but may extend up to 50,000/ μ L (Chapters 122–125). Thrombocytopenia should be ruled out by determining thrombocyte count in any bird suspected of having a hemostatic defect.

SAMPLE COLLECTION FOR COAGULATION TESTING

Blood collection from birds is an art, requiring careful, precise technique. Obtaining valid coagulation results

depends on skillful venipuncture to minimize sample contamination with tissue fluid, collection of blood into citrate anticoagulant, and careful processing to prevent *ex vivo* factor activation (Chapter 138).

Venipuncture Technique

Venipuncture is performed with 25–27 gauge needles to minimize trauma and facilitate post-collection hemostasis. Forcing blood through small-bore needles causes hemolysis; therefore needles should be removed before blood transfer from syringes to processing tubes. The most useful syringe sizes are 3 mL, 1 mL, and 0.5 and 0.3 mL. Large syringes produce excessive negative pressure resulting in vascular collapse, endothelial damage, and hemolysis.

The right jugular, medial metatarsal, and basilic veins are the main vessels used for blood collection. When accessible, the jugular vein is preferred due to its relatively large size. The jugular vein is located dorso-laterally on the neck and is usually much larger on the right. In most birds, gently blowing to part the feathers on the neck exposes bare skin overlying the jugular vein (the jugular furrow or groove). In birds lacking a jugular furrow (e.g. waterfowl), wetting the feathers with alcohol aids in vessel localization. Columbiformes (e.g. pigeons) have a vascular plexus rather than distinct jugular vein, and air sac location in other species (e.g. hornbills) precludes blood collection from the jugular vein. Similarly, fat may obscure the jugular vein in obese birds, especially some Amazon parrot species.

The medial metatarsal vein is an alternate to the jugular; however, its localization may require plucking a few feathers, and venipuncture site hemorrhage may be difficult to control. The basilic (ulnar) vein is readily accessible, but a lack of overlying supportive tissue often results in hematoma formation after venipuncture and vessel immobilization may be impossible in awake birds. Blood collection from a clipped toe-nail is a painful and inappropriate technique that risks sample contamination with tissue fluid or uric acid, and uncontrollable hemorrhage.

Blood Collection and Processing

Approximately 10% of total blood volume (estimated as 0.8–1% of total body mass in grams) can be drawn safely from euvoletic, and nonanemic birds. Blood samples must be collected in sodium citrate anticoagulant; heparin-anticoagulated samples are *invalid* for coagulation testing. For optimal sample quality, blood is drawn into a syringe containing premeasured sodium citrate to obtain an exact ratio of 1 part citrate to 9 parts blood. The sample is then gently mixed, transferred to a tube for centrifugation, and the supernatant plasma removed for same-day assay, or stored frozen (–20 °C). Prolonged storage as whole blood or repeated freeze-thaw cycles may produce artifactually prolonged clotting times due to protease activation and factor/fibrinogen degradation.

TABLE 91.1 Avian Prothrombin Times

Subject	PT Range ^a (s)	Mean PT ^a (s)	Reference
Chicken	9–11	10	5
Chicken (24 weeks)	10–22	15	1
Chicken (8 weeks)	13–27	17	1
	9–15	12	25
Turkey (40 weeks)	10–27	14	1
Turkey (8 weeks)	11–29	17	1
Quail	10–15	12	11
Pigeon	9–21	14	26
Kite	8–19.5	13	26
Vulture	11–17	14	26
Hispaniolan parrot	9–11	10	15
Cockatoo	10–16	12	15

^aPT, prothrombin time.

COAGULATION TESTING

Coagulation screening tests assess factor function based on rate of *in vitro* fibrin formation (see Chapter 138). Due to species and assay variables, evaluating results from paired same-species controls and/or same-species reference intervals facilitates test interpretation.

Prothrombin Time

The prothrombin time (PT) is the most commonly performed coagulation assay in birds.^{1,6,15,25,26} The assay is configured with a thromboplastin/calcium reagent to initiate clotting and is a screening test of the extrinsic and common pathway (factors VII, V, X, prothrombin and fibrinogen). Prolonged PT is compatible with coagulopathy due to vitamin K deficiency, severe hepatic failure, or factor consumption. The source of tissue factor in thromboplastin reagents is a major assay variable affecting PT.^{26,28} Optimized PT assays require avian-origin thromboplastins. Thromboplastins from the species of interest are ideal; however, a commercial chicken-brain extract (Innovative Research, Novi, Michigan, www.innov-research.com) has general use for avian PT. The reported values for avian PT (Table 91.1) reflect species differences, and variability in sample collection, plasma storage, and assay techniques.

Activated Clotting Time

The activated clotting time (ACT) is a point-of-care test that measures the time to clot formation for blood drawn into a particulate activator tube. The assay is an intrinsic and common pathway screening test that detects moderate to severe deficiencies of factors VIII, IX, V, X, prothrombin and fibrinogen. Although the use of PT is commonly reported, the ACT has potential as an in-house screening test.^{1,5} In the authors' experience, quail ACT is less than 3 minutes, similar to human ACT. Whole blood clotting times have been reported for chickens; however, marked variability and wide range

(<1–39 minutes) limit the clinical utility of this screening test.²⁵

Fibrinogen

Heat precipitation techniques provide estimates of fibrinogen concentration to detect hyperfibrinogenemia. The Clauss technique, based on thrombin clotting time of plasma, is a more sensitive, quantitative measure to diagnose hypo- and hyperfibrinogenemia. Hypofibrinogenemia has been described in birds with sepsis-induced disseminated intravascular coagulation (DIC) and hepatic failure.^{6,22} Hyperfibrinogenemia secondary to bacterial infection has been reported in many species, including African gray parrots, cranes, flamingos, and penguins.¹²

AVIAN HEMORRHAGIC DISORDERS

Vitamin K Deficiency

The first reported coagulopathy in birds, characterized by intramuscular, subdural, and ecchymotic hemorrhage, was described in chicks fed a vitamin K-deficient diet.³ Although dietary vitamin K deficiency and intestinal malabsorption due to sulfa drugs are now relatively infrequent,²¹ anticoagulant rodenticide intoxication has become an important vitamin K deficiency syndrome in birds.^{2,13,17,24} Due to high prevalence, rodenticide toxicity should be suspected in birds with hemorrhage, or less specific signs of anemia, hypoproteinemia, and shock. Granivorous birds directly ingest anticoagulants in baits, while predatory birds develop secondary toxicity through ingestion of anticoagulant-poisoned prey. Government agencies from New York to New Zealand have noted the adverse impact of rodenticide use on wild bird populations. Toxicities have occurred in free-ranging species, (e.g. barn owls, eastern screech-owls, great horned owls, red tailed hawks, seabirds) and in captive turkey vultures. Owls appear to be at high risk since small rodents are their primary food source.²⁴ Brodifacoum, a second-generation product with long half-life, is the most commonly implicated poison. The toxic dose of brodifacoum is not defined for most species; however, LD₅₀ values of 0.31 mg/kg have been reported for mallard ducks and 4.5 mg/kg for chickens.¹⁸

Coagulopathy caused by vitamin K antagonists results in deficiency of factors VII, IX, X and prothrombin, which prolongs the PT (Chapter 85). Definitive diagnosis is based on identification of specific poisons in stomach contents, tissues, or bait samples.²⁴ The Louisiana Animal Disease Diagnostic Laboratory and other veterinary toxicology laboratories offer test panels to screen for anticoagulant rodenticides.

Hepatotoxins and Hepatic Disease

Severe liver disease in birds, as in mammals, causes synthetic failure of coagulation factors and fibrinogen.

Aflatoxin, produced by *Aspergillus* sp. fungi, is well-recognized as a potent hepatotoxin that causes fatty liver degeneration in birds and coagulopathy due to hepatic dysfunction.^{5,7,20} Chickens dosed at 5 mg/kg aflatoxin had significantly prolonged PT with no clinical signs; however, species differences in susceptibility exist as turkeys and bobwhite quail are more susceptible than chickens and Japanese quail, respectively.²⁰ Other mycotoxins (e.g. fumonisin) and toxins in plants such as rapeseed may also induce hepatotoxicity and coagulopathy.^{5,6} Metabolic and infectious disease (e.g. hepatic lipidosis, hemochromatosis, Gram negative bacterial infections, mycobacteriosis, chlamydophilosis, and biliary adenocarcinoma associated with viral disease) are additional causes of hepatic failure and coagulopathy in birds.

Fatty liver hemorrhagic syndrome is characterized by fat deposition in the liver and abdomen, and massive abdominal hemorrhage in laying hens consuming large amounts of high-energy diets.²⁷ The syndrome is associated with changes in plasma phospholipid composition, rather than factor deficiencies, and genetic factors are believed to influence susceptibility. Management practices (e.g. feeding nutritionally balanced layer diets and free-range production) appear to prevent the problem.

Infectious Disease

Numerous viral diseases are associated with hemorrhage, including circovirus, reovirus, herpesvirus, polyomavirus, and avian influenza.^{10,16} Retroviral infection has also been proposed as a cause of Conure bleeding syndrome, an ill-defined syndrome characterized by oral or cloacal hemorrhage and regenerative anemia. The pathogenesis of coagulopathy in viral disease is likely multifactorial, including hepatic synthetic failure, vasculitis, and DIC.^{10,12,16}

Pathogens capable of inducing DIC in birds include *Borrelia anserina*, *Salmonella pullorum*, *Escherichia coli*, *Erysipelothrix rhusiopathiae*, adenovirus, infectious bursal disease virus, laryngotracheitis virus, and avian influenza virus.^{16,22,23} Recent studies of highly pathogenic avian influenza (HPAI) in chickens reveal clinical evidence of severe hemorrhage in multiple organs (lungs, gastrointestinal tract, musculature) and histologic evidence of fibrin thrombi in pulmonary arterioles.¹⁶ Molecular analyses support a DIC process initiated by up-regulation of tissue factor expression, in addition to endothelial destruction and inflammatory cytokine release. As in mammals, clinico-pathologic abnormalities aid in antemortem diagnosis of DIC (Chapter 88). Findings supportive of overt DIC include prolonged PT, low thrombocyte count, and low fibrinogen.

TREATMENT GUIDELINES

Vitamin K₁ administration is indicated for vitamin K deficiencies caused by rodenticide toxicity, nutritional

deficiency, antibiotic administration, and recommended prior to hepatic biopsy. Oral vitamin K₁ (phylloquinone; 0.13 mg/kg) is given to poultry as basic nutrition, with demonstrable shortening of PT.¹⁴ Recommended dosages to reverse vitamin K-dependent coagulopathy range from 0.2 to 2.5 mg/kg SC q4–12 hours.¹⁷ Due to the widespread use of long-acting rodenticides (e.g. brodifacoum), vitamin K replacement therapy is typically instituted for at least 1 month. Monitoring PT after discontinuing vitamin K₁ aids in detection of persistent anticoagulant effect, necessitating reinstitution of vitamin K therapy.

Birds with active or severe hemorrhage due to coagulopathy may require transfusion of whole blood to replace both red blood cells and clotting factors. Homologous transfusions are preferred due to risk of acute immune reactions and/or short survival time of heterologous red cells (Chapter 99).

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The background of the page is a microscopic image of a blood smear. It shows a variety of cells: numerous red blood cells (erythrocytes) of different sizes and shapes, some with central pallor; several white blood cells (leukocytes) with distinct nuclei and some granules; and small, dark-staining platelets (thrombocytes).

SECTION VIII

Transfusion Medicine

Mary Beth Callan

Erythrocyte Antigens and Blood Groups

GORDON A. ANDREWS and MARIA CECILIA T. PENEDO

Red Blood Cell Antigens and Blood Groups in the Dog

Historical Background

Nomenclature

Dog Erythrocyte Antigen System

DEA 1.1, 1.2, 1.3 (A system)

Incidence

Molecular identity

Transfusion reactions

Neonatal isoerythrolysis

DEA 3 (B System)

DEA 4 (C System)

DEA 5 (D System)

DEA 6 (F System)

DEA 7 (Tr System)

DEA 8

Other Specificities

Red Blood Cell Antigens and Blood Groups in the Cat

Historical Background

Cat AB Blood Group System

Genetics

Incidence of the antigens

Molecular characterization of the antigens

Molecular genetics

Transfusion reactions

Neonatal isoerythrolysis

Other Specificities

The *Mik* red blood cell antigen

Red Blood Cell Antigens and Blood Groups in

Livestock Species

Historical Background

Methods of Blood Group Detection

Source of blood typing reagents

Hemolytic tests

Agglutination tests

Blood Group Systems

Cattle

Horse

Sheep and goat

Pig

Llama and alpaca

Applications of Blood Groups

Animal breeding

Blood transfusion

Plasma transfusion

Neonatal Isoerythrolysis in Horses

NI disease in mule foals

Red blood cell donors for foals with NI

Acronyms and Abbreviations

CMAH, cytidine monophospho-N-acetylneuraminic acid hydroxylase; DLH, domestic longhair; DSH, domestic shorthair; EA, erythrocyte antigen; FUT1, fucosyl-transferase 1; FUT2, fucosyl-transferase 2; NeuAc, N-acetylneuraminic acid; [NeuAc]2GD3, NeuAc-NeuAc-galactose-glucose-ceramide; NeuGc, N-glycolylneuraminic acid; [NeuGc]2GD3, NeuGc-NeuGc-galactose-glucose-ceramide; NI, neonatal isoerythrolysis; RBC, red blood cell.

Inherited cell surface antigens located on the red blood cell (RBC) membrane are referred to as blood group antigens. Their detection and description are based on polyclonal or monoclonal antibody serology. The antigens can vary in immunogenicity and, therefore, clinical significance. These antigens participate in recognition of self and in some cases can serve as markers of disease. In humans, for example, neoplastic cells sometimes express altered blood group antigens. In veterinary medicine the clinical significance of blood group antigens is usually in the areas of transfusion

reactions and neonatal isoerythrolysis (NI). Blood group antigens are genetic markers and have in the past been used to resolve cases of disputed parentage. Though not yet proven in veterinary medicine, blood group antigens may play a role in immune-mediated hemolytic anemia and may serve as markers of disease. In this chapter our current understanding of the nomenclature, serology, biochemistry, inheritance, incidence, and transfusion significance of the dog, cat, cattle, horse, sheep, goat, pig, llama and alpaca blood groups is reviewed.

RED BLOOD CELL ANTIGENS AND BLOOD GROUPS IN THE DOG

Historical Background

The discovery of the human ABO blood group system in 1900 by Landsteiner stimulated the search for blood group systems in domestic animals. Canine blood groups were first recognized by Von Dungern and Hirszfeld in 1910, who defined four different blood groups based on immune isoagglutinins.⁹⁵ Swisher and coworkers at the University of Rochester, NY, used dogs as animal models to study in vivo mechanisms of RBC destruction. In a series of reports they described antigens A, B, C, D, E, F, and G, determined the frequencies of the antigens in a random population of dogs, and characterized the in vivo and in vitro behavior of the isoantibody systems. Their systematic approach to studying the canine blood group systems was reviewed in 1961 and 1962.^{94,95}

Nomenclature

The blood group antigens were first given letter designations A, B, C, D, E, F, and G in the order of their discovery. Two categories of A cells were recognized, a strongly reactive group (A), and a distinct series of less strongly reacting cells designated as A' that could only be detected by the Coombs' antiglobulin test. This designation was later changed to A1 and A2.⁹⁵ Anti-E and anti-G typing sera were lost, so no future comparisons could be made.^{12,26,93} International workshops met in 1972 and 1974 to standardize canine blood groups as defined by isoimmune sera and to standardize canine blood group system nomenclature.^{101,102} The first workshop designated the terminology canine erythrocyte antigen (CEA) followed by a number to indicate the blood group antigen. The second workshop adopted the designation dog erythrocyte antigen (DEA) followed by a number from 1 onward for a locus, followed by a (.), followed by another number for each allele recognized at a locus. The new terminology was adopted to avoid confusion with the carcinoembryonic antigen (CEA) system.

A nomenclature system has been proposed that conforms with that used in other species.^{26,80} This system would return to an alphabetical designation to name blood group systems (loci), and would also identify factors, alleles, genotypes, and phenotypes. Based on the last international cooperative effort at defining canine blood group antigens,¹⁰¹ general usage among veterinary immunohematologists, and the availability of typing sera, the nomenclature system currently used in the United States is the DEA system which includes DEA 1.1, DEA 1.2, DEA 3, DEA 4, DEA 5, and DEA 7. There is not worldwide acceptance of the DEA nomenclature system, however, and some authors use the newer genetic nomenclature system in reporting new blood group specificities.^{96,97} Until there is a standardized international nomenclature system, it is necessary to be familiar with both systems. The changes in nomen-

clature of the dog blood group systems are summarized in Table 92.1.

Typing sera are commercially available in the United States only for DEA 1.1, 1.2, 3, 4, 5, and 7 (see Chapter 139).⁵² From a practical standpoint, therefore, these are the antigens with which the veterinary practitioner should be most familiar. However, many other blood group factors and systems have been described, and the lack of commercially available typing sera does not diminish the potential significance of these other systems in transfusion medicine.

Dog Erythrocyte Antigen System

DEA 1.1, 1.2, 1.3 (A system)

This system is a three factor, four phenotype system that contains the antigens DEA 1.1, 1.2, 1.3, (equivalent to Aa₁, Aa₂, Aa₃) and a null type.⁹⁶ Breeding studies suggest an autosomal dominant pattern of inheritance with the order of dominance being DEA 1.1, 1.2, 1.3, and null.^{24,94,96} Individual dogs exhibit only one phenotype. The antigens are described as subtypes of a linear series.⁸⁸ A subtypic series has the characteristic that isoimmune antisera produced to one of the antigens can exhibit grades of cross-reactivity with other antigens in the series. Anti-A (anti-DEA 1.1, 1.2, 1.3) antiserum is produced by immunizing an A (DEA 1.1, 1.2, 1.3) negative dog with RBCs from a DEA 1.1 positive dog. Anti-A antiserum strongly agglutinates and hemolyzes (in the presence of fresh complement) RBCs having DEA 1.1 and causes variable agglutination (but not hemolysis) and sensitizes RBCs having DEA 1.2 and 1.3 in the antiglobulin test. Anti-A antiserum is, therefore, a mixture of anti-DEA 1.1, 1.2, and 1.3. Anti-DEA 1.1 antiserum is produced by immunizing a DEA 1.2 positive dog with RBCs from a DEA 1.1 positive dog.^{52,94} Anti-DEA 1.1 antiserum reacts only with RBCs having DEA 1.1. When an A (DEA 1.1, 1.2, 1.3) negative dog is immunized with Aa₃ (DEA 1.3) positive cells, the initial antiserum contains antibodies that strongly agglutinate and hemolyze Aa₁ and Aa₂ (DEA 1.1 and DEA 1.2) cells and weakly agglutinate Aa₃ cells. Repeated exposure to Aa₃ cells results in antisera that are strongly reactive with all three cell types.⁹⁶ Attempts to produce specific anti-DEA 1.2 and anti-DEA 1.3 have been unsuccessful without performing RBC absorption procedures.^{95,96}

Incidence The incidence of DEA 1.1 and DEA 1.2 in the United States is approximately 45% and 20%, respectively.^{52,95} DEA 1.3 has been evaluated only in Australia.

Molecular Identity Immunoprecipitation experiments using polyclonal antisera to DEA 1.2 isolated a protein with a molecular mass of 85 kDa.²⁸ Western blotting experiments using monoclonal antibody specific for DEA 1.1 identified two membrane proteins with molecular mass 50 kDa and 200 kDa.²

Transfusion Reactions The A system is the most important blood group system from a practical stand-

TABLE 92.1 Canine Blood Group System Nomenclature

1951 ^a	1961 ^b	1973 ^c	1973 ^d	1976 ^e	1979 ^f	1980 ^g	1991 ^h	Alleles	Phenotypes	Genotypes
					(proposed)	(proposed)	(proposed)			
					Blood Group System	Blood Factors	Blood Factors			
A	A1	A1	CEA 1	DEA 1.1	A	Aa ₁	Aa ₁	A ^{a1}	A(a ₁)	A ^{a1} A ^{a1} A ^{a1} A ^{a2} A ^{a1} A ^{a3} A ^{a1} A ⁻ A ^{a2} A ^{a2} A ^{a2} A ^{a3} A ^{a2} A ⁻ A ^{a3} A ^{a3} A ^{a3} A ⁻
A'	A2	A2	CEA 2	DEA 1.2	—	Aa	Aa ₂	A ^{a2}	A(a ₂)	A ^{a2} A ^{a2} A ^{a2} A ^{a3} A ^{a2} A ⁻
—	—	—	—	—	—	—	Aa ₃	A ^{a3}	A(a ₃)	A ^{a3} A ^{a3} A ^{a3} A ⁻
B	B	B	CEA 3	DEA 3	B	Ba	Ba	B ^a	B(a)	B ^a B ^a B ^a B ⁻
—	—	—	—	—	—	—	—	B ⁻	B(-)	B ⁻ B ⁻
C	C	C	CEA 4	DEA 4	C	Ca	Ca	C ^a	C(a)	C ^a C ^a C ^a C ⁻
—	—	—	—	—	—	—	—	C ⁻	C(-)	C ⁻ C ⁻
D	D	D	CEA 5	DEA 5	D	Da	Da	D ^a	D(a)	D ^a D ^a D ^a D ⁻
—	—	—	—	—	—	—	—	D ⁻	D(-)	D ⁻ D ⁻
—	E	—	—	—	—	—	—	—	—	—
—	F	F	CEA 6	DEA 6	F	Fa	Fa	F ^a	F(a)	F ^a F ^a F ^a F ⁻
—	—	—	—	—	—	—	—	F ⁻	F(-)	F ⁻ F ⁻
—	G	—	—	—	—	—	—	—	—	—
—	—	Tr	CEA 7	DEa 7	Tr	TR ^{tr}	TR ^{tr}	TR ^{tr}	Tr(tr)	Tr ^{tr} Tr ^{tr} Tr ^{tr} Tr ⁰ Tr ^{tr} Tr ⁻
—	—	—	—	—	—	Tr ⁰	Tr ⁰	Tr ⁰	Tr(O)	Tr ⁰ Tr ⁰ Tr ⁰ Tr ⁻
—	—	—	—	—	—	—	—	Tr ⁻	Tr(-)	Tr ⁻ Tr ⁻
—	—	He	CEA 8	DEA 8	? ⁱ	—	—	—	—	—

^aRef. 23. ^bRef. 95. ^cRef. 93. ^dRef. 102. ^eRef. 101. ^fRef. 80. ^gRef. 26. ^hRef. 96. ⁱWas not designated.

point for transfusion in the dog. Anti-DEA 1.1 is a strong agglutinin and hemolysin in vitro and in vivo. Naturally occurring antibody to DEA 1.1 and 1.2 has not been documented, so first time acute hemolytic transfusion reactions do not occur. However, subsequent incompatible transfusions can result in severe hemolytic transfusion reactions with hemoglobinuria, hyperbilirubinemia and removal of the transfused RBCs in less than 12 hours (see Chapter 100).^{42,111}

Transfusion of plasma containing anti-DEA 1.1 antibody in one study resulted in a hemolytic reaction in DEA 1.1 positive recipients.¹¹¹ DEA 1.1/1.2 negative dogs sensitized with DEA 1.1 positive RBCs will develop antisera that can on subsequent exposure remove transfused DEA 1.2 RBCs in 12–24 hours. DEA 1.2 positive dogs produce a strong anti-DEA 1.1 antibody when exposed to DEA 1.1 RBCs and experience an immediate hemolytic transfusion reaction when subsequently exposed to DEA 1.1 RBCs.⁵² The third factor in this system, DEA 1.3 (or Aa₃) has been described, but transfusion experiments have not been performed.⁹⁶ However, the serological characteristics described

above make the transfusion significance of this antigen obvious.

Neonatal Isoerythrolysis Hemolytic disease in neonatal pups proposed to be naturally occurring NI has been reported.¹¹³ Experimental NI due to blood group A incompatibility has been produced in pups by immunization of an A-negative bitch by transfusion with A-positive RBCs, followed by mating to an A-positive sire.^{22,109,110} Sensitization of a DEA 1.1 negative pregnant bitch by previous transfusion or pregnancy can result in NI in DEA 1.1 positive pups.¹⁰⁹

DEA 3 (B system)

This is currently understood to be a one factor, two phenotype system with the antigen DEA 3 and a null phenotype, with DEA 3 being dominant. Naturally occurring anti-DEA 3 antibody has been reported in up to 20% of DEA 3 negative dogs.^{52,113} In the United States, approximately 6% of the general dog population is DEA 3 positive.⁹⁵ However, 23% of greyhounds typed at

Michigan State University between 1990 and 1995 were DEA 3 positive.⁵² Administration of DEA 3 positive RBCs to a sensitized dog results in loss of the transfused RBCs within 5 days and can result in severe acute transfusion reactions.^{34,94}

In Japan, two reagents, a heteroimmune antiserum produced in rabbits, designated anti-D1, and an isoimmune antiserum, designated anti-E, were found in comparison tests with DEA reagents to be identical to anti-DEA 3.³¹ Western blotting experiments using monoclonal antibody to DEA 3 identify five bands with molecular mass of 34, 53, 59, 64, and 71 kDa.⁵⁴

DEA 4 (C system)

This is a one factor, two phenotype system with the antigen DEA 4 and a null phenotype, with DEA 4 being dominant. Naturally occurring antibody to DEA 4 has not been documented. Up to 98% of the general dog population in the United States expresses this antigen.⁹⁴ However, the incidence of DEA 4 may be significantly lower in some breeds and geographic locations.⁵² DEA 4 negative dogs will produce antibody to this antigen when exposed by transfusion, but sensitized dogs did not show RBC loss or hemolysis when transfused with DEA 4 positive cells.⁹⁴ Attempts to induce NI with blood group C (DEA 4) incompatibility were unsuccessful.¹¹² However, a hemolytic transfusion reaction is described in a DEA 4 negative patient that received multiple transfusions of DEA 4 positive RBCs.⁷⁰ The transfusion significance of this antigen is considered incomplete.⁵² Immunoprecipitation experiments using polyclonal antisera to DEA 4 isolated a protein of molecular mass 32–40 kDa.²⁸

DEA 5 (D system)

This is a one factor, two phenotype system with the antigen DEA 5 and a null type, with DEA 5 being dominant. Naturally occurring antibody to this antigen has been reported in 10% of random non-transfused dogs in the United States.^{94,113} The incidence of this antigen is generally low, but there is breed and geographic variation. Up to 30% of greyhounds in the United States are DEA 5 positive.⁵² Transfusion of DEA 5 positive RBCs to sensitized dogs results in RBC sequestration and loss within 3 days.⁹⁴ Experimental attempts to induce NI with blood group D (DEA 5) incompatibility were unsuccessful.¹¹²

DEA 6 (F system)

This is a one factor, two phenotype system with the antigen DEA 6 and a null phenotype, with DEA 6 being dominant. Although reported in almost 100% of dogs in the United States,⁹⁵ this antigen also exhibits marked breed and geographic variation in expression.^{33,53,102} Naturally occurring anti-DEA 6 has not been documented. Transfusion studies in one DEA 6-negative dog showed moderately rapid removal of transfused RBCs

following sensitization.⁹⁵ Typing sera for DEA 6 no longer exists.

DEA 7 (Tr system)

The Tr antigen antibody system was defined by a naturally occurring isoantibody and by an identically reacting antiserum produced by isoimmunization.¹⁶ Factor O is an allele in the Tr system, resulting in the Tr system being a two factor, three phenotype (Tr, O, and null) system with the order of dominance being Tr, O, and null.²⁷ The Tr antigen is not an integral RBC membrane antigen, but rather is believed to be produced elsewhere in the body, secreted into the plasma, and adsorbed onto the RBC surface.²⁰ The Tr antigen is expressed in approximately 40–54% of dogs.^{16,26,102} Naturally occurring, weak, low titered, nonhemolytic anti-DEA 7 is present in 20–50% of DEA 7-negative dogs, and sensitized DEA 7-negative dogs, when transfused with DEA 7-positive RBCs show sequestration and loss of RBCs within 72 hours.⁵² Immunoprecipitation experiments using polyclonal antisera to DEA 7 isolated three proteins of molecular mass 53, 58, and 66 kDa.²⁸

DEA 8

This canine RBC antigen, originally designated as He, was discovered by antiserum raised by isoimmunization, and the antigen reported to exist in 40–45% of a random population of dogs.¹⁹ Typing sera for DEA 8 no longer exists.

Other Specificities

N-Acetyl-neuraminic acid (NeuAc) and *N*-glycolyl-neuraminic acid (NeuGc) present on gangliosides (hematosides) of the RBC membrane define an unnamed blood group system described in Japan.^{55,107} Sera from dogs with NeuAc hematoside contain an isoantibody that agglutinates RBCs of dogs with NeuGc hematoside, and the agglutination is inhibited by NeuGc hematoside. Comparison studies with standardized canine blood typing reagents have not been performed. Several Oriental breeds express NeuGc hematoside, especially native breeds of northern China, Korea, and southern Japan, including the Japanese mongrel, Kai, Kishu, Japanese spaniels, and Shiba dogs.

A blood group system referred to as the D system is described in Japan. The D system is composed of two antigens, D1 and D2, with three phenotypes, D1, D2, and D1D2. The phenotypes are based on agglutination testing with two rabbit heteroimmune antisera, anti-D1 and anti-D2. The D1 and D2 antigens are codominant factors and anti-D1 is identical to anti-DEA3.³¹ The counterpart of D2 has not been described outside of Japan. The incidence of D1 and DEA 3 is high in purebred dogs indigenous to Japan such as the Akita, Shiba, Kishu, Shikoku, and native Japanese mongrel and mixed breeds, while most Western and European breeds are negative for these factors.^{31,33,34} Repeated transfusion

of D2 type blood into a D1 type patient, or of D1 type blood into a D2 type patient results in severe acute transfusion reactions, indicating the importance of this system in transfusion medicine.³⁴

The lectin extracted from seeds of *Clerodendron tricotomum* preferentially agglutinates RBCs of some dogs (designated type C) at titers up to 128, and is completely negative for other dogs (type c).¹⁰⁸ Genetic studies in Beagles (62 offspring from 18 matings) indicated that the characteristic is inherited as an autosomal dominant trait.⁶⁴ This system, designated as "C", was compared to the DEA system and found to be different.^{3,31,62,64}

The *Dal* blood type is defined by specific IgG alloantibodies in a Dalmatian dog previously sensitized by blood transfusion.¹⁵ Major crossmatching tests with 55 non-Dalmatian dogs were incompatible, suggesting the antibody recognized a common RBC antigen. Four of 25 unrelated Dalmatians were crossmatch compatible, suggesting that they lacked the antigen. Blood typing of the recipient and donors indicated that the alloantibodies were not associated with DEA 1.1, 1.2, 3, 4, 5, or 7.

Numerous other specificities have been reported and proposed to represent new blood group systems.^{26,31,92,97,101}

RED BLOOD CELL ANTIGENS AND BLOOD GROUPS IN THE CAT

Historical Background

Ingelbringsten (cited by Ottenburg and Thalhimer)⁷⁵ first described naturally occurring isoagglutinins in cats, but did not find evidence of groups similar to the human ABO types. Holmes reported two groups of cats, one designated as group O that contained an isoagglutinin in the serum, and a group with the corresponding antigen on the RBCs named group EF.⁵⁸ Eyquem described two antigens, A and B, and stated that these antigens were identical to EF and O reported by Holmes.³⁵

Auer and Bell used two naturally occurring antibodies, anti-A and anti-B, to characterize the cat blood group system which they designated as AB, and first reported the rare AB phenotype where both antigens were present on the RBC.⁷ They were also the first to describe immediate experimental and naturally occurring transfusion reactions in previously unsensitized cats of known incompatible blood type.⁹

Cat AB Blood Group System

The AB blood group system characterized by Auer and Bell is the predominant blood group system in cats.⁷ Three phenotypes occur: type A, type B, and type AB. A null phenotype does not occur.

The types are defined by naturally occurring isoantibody against the antigen they lack. Type A cats have low titered anti-B hemagglutinins of the IgM class and hemolysins consisting of equal amounts of IgG and

IgM.¹⁸ Approximately one-third of type A cats have macroscopic agglutinins and hemolysins. Microscopic examination or the antiglobulin test is required to detect the weak agglutination reactions in the remaining two-thirds of the cats. Type B cats have high titered anti-A hemagglutinins and hemolysins mainly of the IgM class with lesser amounts of IgG.^{18,104} It is the presence of these naturally occurring isoantibodies that is responsible for transfusion reactions and NI in blood type incompatible cats. Type AB cats do not possess isoantibodies against either A or B antigens.

Genetics

The blood group antigens A and B are inherited as simple autosomal Mendelian traits with A being dominant to B.^{12,41} All type-B cats are homozygous for the B allele (genotype B/B) and type-A cats can be either homozygous (genotype A/A) or heterozygous (genotype A/B). Blood type AB is not the result of codominant inheritance of type A and B, nor is it the result of chimerism.⁷ In spite of extensive breeding studies and pedigree analysis, the mode of inheritance of the AB phenotype remains unknown, although it appears to be recessive to type A and dominant to type B.^{7,47}

Incidence of the Antigens

Type A is the most common blood type. Type B is less common, and type AB is very rare. The percentage distribution of types A and B in domestic shorthair (DSH) and domestic longhair cats (DLH) can vary markedly with geographic location within the United States and worldwide (Table 92.2). The breed incidence of type B in the United States ranges from 0% up to 60% among different purebred cats (Table 92.3).

In Turkey, 60% of Van cats and 46.4% of Angora cats are type B.⁵ Type AB is exceedingly rare. In a survey of cats in the United States and Canada, 13 of 9,239 cats (0.14%) were type AB.⁴⁷ In Australia, 7 of 1895 cats (0.4%) were type AB.⁷ Type AB occurs only in breeds in which type B is detected. Type AB has been found in DSH/DLH cats and in Abyssinian, Birman, British Shorthair, Norwegian Forest, Persian, Scottish Fold, Cornish Rex, Devon Rex, Maine Coon, Manx, Ragdoll, Sphynx, Bengal, Egyptian Mau, Siberian, European, and Somali purebred cats.^{13,47}

Molecular Characterization of the Antigens

The form of neuraminic acid (sialic acid) on the RBC membrane glycolipids and glycoproteins determines the blood group antigens in the cat.^{4,50} NeuGc is the determinant of the A antigen. Type-A RBCs have NeuGc-NeuGc-galactose-glucose-ceramide ([NeuGc]₂G_{D3}) as the major disialoganglioside, and it is the predominant blood type-determining ganglioside detected by feline anti-A antisera.⁴ Type A RBCs also have NeuAc-NeuGc-G_{D3}, NeuGc-NeuAc-G_{D3}, and a very minor amount of [NeuAc]₂G_{D3}. Homozygous and

TABLE 92.2 Blood Type Frequencies in DSH/DLH and Non-Pedigree Cats

Country	n	A (%)	B (%)	AB (%)	Reference
Australia	1895	73.3	26.3	0.4	7
Australia	355	62.0	36.0	1.6	68
Austria	101	97.0	3.0	0.0	43
Denmark	105	98.1	1.9	0.0	65
England	105	67.6	30.5	1.9	38
England	477	97.0	3.0	0.0	58
Finland	61	100.0	0.0	0.0	43
France	350	85.0	15.0	0.0	35
Germany	600	94.0	6.0	0.0	43
Germany (DSH/DHL)	404	94.1	5.9	0.0	51
Germany (Total cats)	868	92.6	6.7	0.7	51
Greece	207	78.3	20.3	1.4	72
Hungary	73	100.0	0.0	0.0	10
Italy	401	88.8	11.2	0.0	43
Japan	207	90.3	9.7	0.0	63
Japan	220	90.0	10.0	0.0	57
Japan	299	89.3	1.0	9.7	32
Portugal	159	89.3	4.4	6.3	83
Scotland	70	97.1	2.9	0.0	59
Spain (Barcelona)	100	94.0	5.0	1.0	79
Spain (Gran Canaria)	97	88.7	7.2	4.1	84
Switzerland	1014	99.6	0.4	0.0	59
Turkey	301	73.1	24.6	2.3	6
USA (by region)					
Northeast	1450	99.7	0.3	0.0	44
North Central/Rocky Mountain	506	99.4	0.4	0.2	44
Southeast	534	98.5	1.5	0.0	44
Southwest	483	97.5	2.5	0.0	44
West Coast	812	94.8	4.7	0.5	44
Virgin Gorda	32	100.0	0.0	0.0	14

TABLE 92.3 Frequency of Blood Type B in Purebred Cats in the United States^a

Type B Frequency (%)	Breeds
0	Siamese, Burmese, Tonkinese, Russian Blue, Ocicat, Oriental Shorthair
<5	Maine Coon, Norwegian Forest, DSH/DLH
5–25	Abyssinian, Himalayan, Birman, Japanese Bobtail, Persian, Somali, Sphinx, Scottish Fold
25–50	Exotic and British Shorthair, Cornish Rex, Devon Rex

^aData compiled from refs. 41, 44, and 48.

heterozygous type A cats have some differences in their ganglioside profiles, and these differences can be used to determine the genotype of type A cats.⁵⁰

NeuAc is the determinant of the B antigen. Type B RBCs have [NeuAc]₂G_{D3} as the only form of the disialo-

ganglioside. Type B RBCs do not contain any detectable NeuGc. Agglutination testing with the lectin of *Triticum vulgare*, a lectin that binds sialoglycoproteins containing NeuAc, demonstrates selective agglutination of type B RBCs.²¹ Type AB RBCs have features of both type A and type B. They are agglutinated by both anti-A and anti-B feline antisera, and by the lectin of *Triticum vulgare* (see Chapter 139). Data from two studies using a panel of murine monoclonal anti-feline blood type A RBC antibodies in agglutination testing, ganglioside thin layer chromatography immunostaining, and flow cytometry suggest that there is more than one biochemical phenotype in the AB type.^{45,46} This phenotypic difference is undetected by feline typing sera, which may explain the reason that investigators have been unable to provide a single genetic mechanism for the inheritance of the AB type.^{4,47,50} Wild felids have the same AB blood group system, and thin layer chromatography of RBC membranes reveals similar ganglioside patterns in wild cats as reported in domestic cats.⁴⁹

Molecular Genetics

Cytidine monophospho-*N*-acetylneuraminic acid hydroxylase (CMAH) is the enzyme that catalyzes the conversion of NeuAc to NeuGc.⁷¹ DNA sequence analysis of the feline CMAH gene revealed several mutations, including two single nucleotide polymorphisms upstream of the start, an indel (insertion/deletion) site in the exon 1 5'-untranslated region, and three missense mutations in the coding region which are concordant with the type A and type B blood types of 18 breeds of cats from the USA and Europe.¹³ These six mutations were homozygous in all type B cats and heterozygous in all heterozygous type A cats. No type A cats were homozygous for any of these six mutations. No concordance was found between the mutations and the blood type AB. This study suggests that the cat AB blood group system resulted from mutation(s) in CMAH that prevent the conversion of NeuAc (blood type B) to NeuGc (blood type A). The authors proposed renaming of the cat AB blood group system to contain three alleles, represented as A>a^{ab}>b. The allele A encodes the wild-type, fully active enzyme, a^{ab} the partially active enzyme, and b the inactive enzyme. Possible genotypes/phenotypes would be AA (type A); Aa^{ab} (type A); Ab (type A); a^{ab}b (type AB); a^{ab}a^{ab} (type AB), and bb (Type B).¹³

Transfusion Reactions

The presence of naturally occurring isoantibodies is responsible for transfusion reactions in previously unsensitized cats (see Chapter 100). Virtually all type B cats have highly titered anti-A agglutinins and hemolysins which can result in severe transfusion reaction and death when as little as 1 mL of type A blood is administered to a type B-cat.^{8,9,39,105} Rapid intravascular destruction of the type A RBCs occurs within minutes to hours and is complement- and IgM-mediated. Type B RBCs administered to type A cats have a half-life of

about 2 days, and minor transfusion reactions can occur.⁸ Removal of type B RBCs occurs mostly extravascularly, involving IgG and IgM without marked complement activity.⁴⁰

Neonatal Isoerythrolysis

Neonatal isoerythrolysis occurs in type A or type AB kittens born to a type B queen.⁴⁷ The colostrum of type B queens contains high concentrations of anti-A antibodies, and colostrum antibody is detectable in the serum of newborns as early as 4 hours after birth.¹⁸ Clinical signs in affected kittens can be variable, ranging from unapparent, to severe hemolytic anemia with hemoglobinuria, icterus, and death. Neonatal isoerythrolysis is believed to be a major cause of the “fading kitten syndrome.”⁴¹

Other Specificities

The *Mik* Red Blood Cell Antigen

This is a RBC antigen discovered by a crossmatch incompatibility between plasma from a feline renal transplant recipient and RBCs from a donor cat which were blood group AB compatible, but resulted in an acute hemolytic transfusion reaction. Plasma from three of 65 previously untransfused blood donor cats were also found to have naturally occurring alloantibody to this common antigen. Plasma from these three donor cats agglutinated RBCs from cats with type A, type B, and type AB blood, indicating the antigen is not part of the AB blood system. The name of the blood group system was designated *Mik* based upon identification of the alloantibody in the first blood donor investigated, a cat named Mike.¹⁰³ The mode of inheritance, frequency of the antigen, geographical distribution, and molecular characterization of the antigen remain to be determined.

RED BLOOD CELL ANTIGENS AND BLOOD GROUPS IN LIVESTOCK SPECIES

Historical Background

The identification of blood group antigens and analysis of their patterns of genetic inheritance in farm animal species were the subject of intense investigation for about 50 years. In contrast to humans and cats, naturally-occurring antibodies are seldom found in the sera of cattle, horse, sheep, goat, pig, llama and alpacas. Production of iso- and heteroimmune antisera by immunization and the use of RBC absorption procedures to isolate monospecific antibodies (blood typing reagents) were critical to uncover the genetic diversity of blood groups in these species. The molecular nature and function of the RBC antigens in farm animals remains largely unknown.

This section describes the blood group systems (or loci) of six livestock species: cow, horse, sheep, goat, pig

and llama (and the related alpaca). Although blood group systems have historically been designated by a single letter, the loci are referred to here by the more recent nomenclature convention in which the two letters “EA” for *erythrocyte antigen* precede the traditional system designation. The literature in blood groups of livestock species is extensive and cannot be fully represented in this section. Excellent reviews of the subject are published that offer historical accounts and details of the complex serological intricacies of blood group loci in the major farm animal species. Readers are referred to the publications by Stormont,⁸⁷ Bell,¹² Di Stasio,³⁰ Hines,⁵⁶ Juneja & Vogeli,⁶⁶ and Sandberg & Cothran.⁸¹ The greatest impact of blood group variation in farm animals was in the area of animal identification and parentage verification, because of the high level of individual discrimination that could be achieved and their classic Mendelian modes of inheritance. From a clinical perspective, knowledge of blood groups is significant only in horses because of the risk of NI posed by maternal-fetal incompatibility for certain factors.

Methods of Blood Group Detection

Hemolytic and agglutination techniques are used to assay blood group factors. The choice of procedure is largely determined by the characteristics of the RBCs of each species. Blood grouping assays for cattle, sheep, goat, llama and alpaca are hemolytic tests because the RBCs of these species are not prone to agglutination. Horse and pig blood groups factors are assayed by saline agglutination or by hemolytic tests. Some pig factors require an antiglobulin test (Coombs’ test) or addition of dextran to facilitate agglutination and improve scoring of reactions.

Source of Blood Typing Reagents

Blood typing reagents (monospecific antisera) for livestock species are not commercially available. They have been traditionally prepared in laboratories that provided blood typing services. Comparison tests sponsored by the International Society of Animal Genetics for several decades provided a forum for standardization of blood factor specificities and nomenclature for each species. Blood typing comparison tests are no longer held and production of blood typing reagents has virtually ceased worldwide with the advent of DNA typing methods and technologies in the late 1990s.

Hemolytic Tests

Hemolytic tests are typically set up in round-bottom, 96-well microtiter plates. A standard procedure consists of combining, in order, 50 μ L of blood typing reagent with 25 μ L of a 2–2.5% saline suspension of washed, packed RBCs and 25 μ L of undiluted rabbit complement. After addition of complement, the plate is shaken in a vibrating plate mixer. Reactions are read twice to record degree of hemolysis: once 30–45 minutes after

set-up and a second time 3 hours later. A concave mirror is used for visual determination of hemolysis which is graded as 0 (negative, no visible hemolysis), 1 (partial hemolysis), 2 (intermediate), 3 (strong, almost complete) and 4 (complete hemolysis). In negative reactions, RBCs remain intact and settle in a pellet at the bottom of the well, whereas with complete hemolysis intact RBCs are not visible and the reaction fluid is clear and reddish in color. A complement control consisting of 50 μ L of saline, 25 μ L of RBC suspension and 25 μ L of complement is run in parallel with the test reactions.

Rabbit serum is the source of complement for hemolytic tests. Because rabbits lack the heterophile Forssman antigen, their sera can have Forssman antibodies in high titers. Cattle and camelids are Forssman-negative and thus hemolytic tests in these species are not affected by Forssman antibodies. For the Forssman-positive horse, sheep, goat and pig, rabbit serum needs to be absorbed with RBCs from these species prior to use as complement.⁹⁰ Two serial absorptions, carried out at 4°C, for 15–20 minutes each and a 1:2 volume ratio of washed, packed RBCs to rabbit serum are usually sufficient to remove heterophile antibodies without affecting complement function.

Agglutination Tests

Saline agglutination tests are set up in the same way as hemolytic tests, except that complement is omitted. Reactions proceed at room temperature for 2–3 hours after which time the plates are then shaken briefly to loosen cells from the bottom of the wells. Degrees of agglutination are recorded 5–10 minutes after resuspension as 0 (no agglutination), 1 (partial clumping), 2 (intermediate clumping) and 3 (total clumping of red cells). A microscope can be used to help distinguish between negative and weak positive reactions.

Antiglobulin tests are used with incomplete antibodies that require the addition of rabbit antiglobulin serum to produce agglutination. This procedure is used to detect some blood group factors in pigs. Addition of dextran to a saline agglutination reaction at a final concentration of 1.5% has been used with some incomplete antibodies in pigs as an alternative to the antiglobulin test.

Blood Group Systems

Cattle

The blood groups of cattle were the first to be studied in detail and provided a model for investigation in other species. The early studies in the 1940s demonstrated the value of iso- and heteroimmunizations for development of antibodies for blood group factors and established the procedures for preparation of monospecific antibodies through absorption and for assaying blood groups with hemolytic tests.^{36,37}

Cattle blood groups remain the primary example of extreme genetic variation of RBC antigens, as evidenced

by more than 70 blood group factors internationally recognized and by the extensive allelic series known in the complex *EAB* and *EAC* systems. An alphabetical nomenclature designates blood group factors (e.g. A, L, S, Z) with numerical subscripts added to describe serologically related subtypes (e.g. A₁, A₂, Y₁, Y₂). The inclusion of the symbols ' and " to a letter (e.g. A', B', A", G") indicates second and third rounds of use of alphabet letters, respectively. One of the most important contributions of the early cattle blood group research was the demonstration that multiple factors segregated together in specific combinations each of which defined an allelic variant.⁸⁹ The term "phenogroup" was coined to describe the combinations of factors.⁸⁶ As reviewed by Hines,⁵⁶ evidence from infrequent recombination events within *EAB* and *EAC* systems suggests that the molecular nature of these complex systems may involve a series of linked genes, each of which could result in products carrying multiple epitopes. With the completion of the bovine genome sequencing project in 2007, the tools are now at hand to investigate the molecular genetic basis of cattle blood group systems. It is of interest to note that the *EAI* system is not an intrinsic component of the RBC membrane and anti-J is the only antibody that occurs naturally. The J factor is a tissue and serum antigen that attaches to RBCs as serum concentrations become sufficiently high. The attached J substance has been shown to be a glycolipid, while the serum form occurs as both glycolipid and glycoprotein.⁹⁸ The J factor is serologically related to human A, sheep R and pig A factors. Cattle blood group loci have been mapped to chromosomes by linkage analysis.⁶⁷ A summary of the 11 cattle blood group loci, factors and allelic variants is given in Table 92.4.

Horse

The first 16 blood group factors in the horse were identified through iso- and heteroimmunization followed by hemagglutination and hemolysis testing.⁹¹ Research by laboratories worldwide led to the subsequent characterization of 34 factors distributed in seven systems. A summary of horse blood group systems, factors and alleles are presented in Table 92.5. By convention, factors within a system are assigned lower case letters and blood group alleles (phenogroups) are designated by the capital letter for the system, followed by lower case letters for the factor or factors detected.

The horse is the only livestock species for which blood group incompatibilities are clinically relevant. Blood group antibodies that cause transfusion reactions or NI can be found in the sera of horses either "naturally" or as a result of a blood group incompatible pregnancy. The majority of analyzed cases of NI are explained by incompatibilities of factors between stallion and mare at either *EAA* or *EAQ*. Naturally occurring anti-Aa (in any of its allelic combinations) and anti-Ac are occasionally found in the sera of horses lacking factor Aa, usually as agglutinins. Anti-Aa in mares may also be present as a hemolysin, particularly

TABLE 92.4 Cattle Blood Group Loci, Chromosome Assignment (Chr), Factors and Alleles (Phenogroups) Defined

Locus	Chr ^a	Blood factors	Number of Alleles	Alleles ^b
EAA	15	A ₁ A ₂ H D Z'	>10	-, A ₁ , A ₂ , A ₁ D, H, A ₁ H, A ₁ Z', etc.
EAB	12	B ₁ B ₂ G ₁ G ₂ G ₃ K I ₁ I ₂ O ₁ O ₂ O ₃ Ox P ₁ P ₂ Q T ₁ T ₂ Y ₁ Y ₂ A' B' D'E' ₁ E' ₂ E' ₃ F' G' I' I' ₂ J' ₁ J' ₂ K' O' P' Q' Y' A'' B'' G'' I''	>1000	-, B ₁ GK ₀ xY ₂ A'O'A'', B ₁ O ₁ , B ₁ O ₁ Y ₂ D', B ₁ I ₁ Q, B ₂ GOx A'A'', O ₁ Q', I ₁ Y ₂ E' ₁ Y', I ₁ Q', Y ₁ D'I', GY ₂ E' ₁ Q', O ₁ T ₁ E' ₃ F'K', O ₁ T ₁ E' ₃ F'G'K'G'', OxQA'E' ₁ O', etc.
EAC	18	C ₁ C ₂ E R ₁ R ₂ W X ₁ X ₂ C' L'X' C''	>60	-, C ₁ , C ₁ EW, C ₁ R ₁ W, C ₁ WL', C ₂ , C ₂ WX ₂ , R ₁ WL', R ₂ WX ₂ L', WX ₂ , etc.
EAF	17	F ₁ F ₂ V ₁ V ₂ N'	7	F ₁ , F ₂ , FN', V ₁ , V ₂ , V ₁ N'
EAJ	11	J	2	-, J
-EAL	3	L	2	-, L
EAM	23	M ₁ M ₂ M'	3	-, M ₁ , M'
EAS	21	S H' U ₁ U ₂ U' ₁ U' ₂ S'' H'' U''	>15	-, SH, H', U ₁ , U ₁ H''H'', U', U'', U''U ₁ H''H'', U' ₂ U'', etc.
EAZ	10	Z	2	-, Z
EAR ^r	16	R' S'	2	R', S'
EAT ^r	19	T'	2	-, T'

^aFrom refs. 61 and 67.

^bThe absence of detectable factors (null allele, recessive) in any locus is represented by a dash (-).

TABLE 92.5 Horse Blood Group Loci, Chromosome Assignment (Chr), Factors and Alleles (Phenogroups) Defined

Locus	Chr ^a	Factors	Recognized Alleles ^b
EAA	20	a b c d e f g	A ^a A ^{adf} A ^{adg} A ^{abdf} A ^{abdg} A ^b A ^{bc} A ^{bce} A ^c A ^{ce} A ^e A ⁻
EAC	Unknown	a	C ^a C ⁻
EAD	14	a b c d e f g h i k l m n o p q r	D ^{adl} D ^{adlnr} D ^{adlr} D ^{bcmq} D ^{cefgmq} D ^{ceginmq} D ^{cefgkm} D ^{cfmqm} D ^{cgm} D ^{cgmp} D ^{cgmq} D ^{cgmr} D ^{cgmr} D ^{dcklr} D ^{deloq} D ^{delaq} D ^{dfklr} D ^{dghmp} D ^{dghmq} D ^{dghmq} D ^{dkl} D ^{dlnq} D ^{dlnq} D ^{dlnq} D ^q (D ⁻)
EAK	2	a	K ^a K ⁻
EAP	Unknown	a b c d	P ^a P ^{ac} P ^{acd} P ^{ad} P ^b P ^{bd} P ^d P ⁻
EAQ	8	a b c	Q ^{abc} Q ^{ac} Q ^a Q ^b Q ^c Q ⁻
EAU	24	a	U ^a U ⁻

^aFrom ref. 76.

^bThe absence of detectable factors is designated by the system letter followed by a dash (-).

if produced by a blood group incompatible pregnancy. Hemolytic screening for this antibody (and others) prior to foaling can provide an effective test to identify mares whose foals are at risk for NI. In EAQ sensitization, the mare is negative for factor Qa (for most breeds this means negative for Q^{abc}), while the stallion has this blood group. The detectable antibody response for Qa has only been reported as a lysin and would be missed by antibody screening based solely on agglutination testing. Occasionally the antibody presence can be detected only very late in pregnancy. Antibodies for blood group factors in EAC, EAD, EAK, EAP and EAU pose little risk as they do not occur naturally and have only rarely been implicated in NI cases, possibly as a result of blood incompatible pregnancies.

Five blood group loci have been mapped to chromosomes by linkage analysis.⁷⁶ A summary of the seven horse blood group loci, factors and allelic variants is given in Table 92.5.

Sheep and Goat

Eight blood group systems have been described in sheep: EAA, EAB, EAC, EAD, EAM, EAR, EAF30 and

EAF41.⁷⁴ The nomenclature of systems and factors follows the same rules as those used for the horse and pig. Although many antigen specificities have been identified, only 22 blood group factors are internationally recognized in sheep. Hemolytic tests are the method of choice for sheep blood group factors with exception of the D system whose antigens are detected by agglutination tests. The sheep EAB, EAC and EAR systems are homologous to cattle EAB, EAC and EAJ. Products of the EAR locus are not an intrinsic component of the RBC membrane. The antigens are soluble substances found in the serum and saliva which attach to the RBC membrane. The EAR^R allele is dominant to EAR^O and expression of both antigens is under the control of the suppressor gene I. Sheep with genotype *i/i* do not express R or O antigens in RBCs, serum or saliva. The EAC locus is closely linked to the Amino Acid Transport gene. This association is of interest because RBCs with defective transport are never Cb-negative. The EAM locus is associated with RBC potassium transport such that low-potassium cells are always Mb-positive and high-potassium cells are always Mb-negative. It has been postulated that Mb inhibits active potassium transport into cells.¹⁰⁰ Several sheep blood group

TABLE 92.6 Sheep Blood Group Loci, Chromosome Assignment (Chr), Factors and Alleles (Phenogroups) Defined

Loci	Chr ^a	Blood factors	Number of Alleles	Alleles ^b
EAA	6	a b	3	–, a, b
EAB	10	a b c d e f g h i	>50 ²	–, a, ab, abc, etc
EAC	20	a b	4 ²	–, a, ab, b
EAD	Unknown	a b	2	–, a
EAM	18	a b c	4	–, a, b, ac, c
EAR	Unknown	R O	2	R, O
EAX	Unknown	X Z	2	X, Z

^aFrom ref. 29.

^bNumber of alleles based on internationally recognized factors.

Additional factors with provisional designations have been identified that increase number to 100 EAB and 20 EAC alleles; the absence of detectable factors (null allele, recessive) in any locus is represented by a dash (–).

systems have been mapped by linkage analysis. A summary of sheep blood group loci, factors and allelic variants is given in Table 92.6.

Blood groups in goats are less developed than those of other farm animals. Six genetic systems have been identified and named EAA, EAB, EAC, EAE, EAF and EAR.⁷³ Several goat blood group factors cross-react with sheep blood typing reagents. The EAA system has a single specificity A1 that is homologous to sheep EAAa. In the EAB system, 14 specificities were identified, four of which are homologous to sheep factors Bb, Be, Bd and Bi. The EAC system has a single specificity related to sheep EACa. The EAE system contains two factors E6 and E18 and the EAF system is defined by a single specificity F19. The EAR system is defined by a single specificity R related to sheep R.

Pig

Development and expansion of pig blood groups is largely due to work carried out in Denmark, Germany, Poland and Russia. The source of blood typing reagents is primarily from isoimmune sera with most antibodies behaving as agglutinins and a few as hemolysins. Sixteen genetic systems are internationally recognized: EAA, EAB, EAC, EAD, EAE, EAF, EAG, EAH, EAI, EAJ, EAK, EAL, EAM, EAN, EAO and EAP.⁶⁶ Some of these, e.g. EAE and EAM, approach the EAB and EAC systems of cattle in complexity and diversity. The nomenclature of pig blood group factors follows that of the horse, except for the EAA system.

The EAA system is related to cattle EAJ, human A and sheep EAR systems. Pig A and O factors occur as soluble substances in the serum and saliva of A-positive and O-positive animals, and attach to the RBC membrane a few weeks after birth. The genetic inheritance of A and O factors is similar to sheep EAR system. EAA^A is dominant to EAA^O and their expression on RBCs is controlled by the suppressor gene, EAS. The EAA^A allele codes for uridine diphosphate N-

acetylgalactosamine transferase that adds a terminal fucose residue to the H substance.⁶⁹ The recessive EAA^O allele contains a deletion within the gene and results in a non-functional enzyme product.¹⁰⁶ The EAH and EAS systems correspond to *fucosyl-transferase 1 (FUT1)* and *fucosyl-transferase 2 (FUT2)* that are involved in the production of the H substance and are related to human H (*FUT1*) and *Secretor (FUT2)* genes.^{25,69} Antigens of the EAN system also occur as soluble substances in the serum and milk but are not secreted into the saliva.

Blood group loci have been mapped to pig chromosomes by linkage analysis.⁷⁸ A summary of pig blood group systems, factors and alleles is given in Table 92.7.

Llama and Alpaca

Little is known about blood group variation in the two domestic South American camelids, llama and alpaca. From iso- and heteroimmune sera developed for these animals, six blood group factors were identified and given alphabetical designations in order of discovery: A, B, C, D, E and F.⁷⁷ Factors A and B are inherited as co-dominant alleles and assigned to blood group system EAA. Factors C, D, E and F were assigned to four separate systems as they appeared to be transmitted independently from each other and from the EAA system. Blood groups in llamas and alpacas are detected by hemolytic tests.

Applications of Blood Groups

Animal Breeding

Accurate pedigree records are essential to many aspects of animal breeding, including selection of breeding stock, estimation of heritabilities, and breeding values based on progeny testing. Most breed registries throughout the world have mandatory parentage testing programs to validate pedigree records of registered animals. From the early 1950s through the mid 1990s, serological tests for blood groups, supplemented by electrophoretic assays that detected additional genetic variation in blood proteins, were the only methods available to verify parentage and to help breeders solve problems of questionable paternity or maternity. The high degree of variation of blood group antigens and protein variants resulted in probabilities of exclusion of incorrect parentage as high as 98% for most breeds.¹⁷ The use of blood typing tests in animal breeding came to an end in the mid 1990s when more powerful and cost effective DNA typing technologies emerged and became established as the preferred genetic testing method for applied and research purposes.

Blood Transfusion

Clinically, knowledge of blood groups in cattle, sheep, goat, pig and llama/alpaca has not affected conventional practice in transfusion. Large animals are not commonly transfused, and matching of blood types for antigenic compatibility is not practical because of the high variability of blood groups between individuals

TABLE 92.7 Sixteen Loci of Pig Blood Groups, Chromosome Assignment (Chr), Test Method, Factors and Alleles (Phenogroups) Defined

Locus	Chr ^a	Test Method ^b	Blood factors	Number of Alleles	Alleles ^c
<i>EAA</i>	1	a, h	A O	2	<i>A, O</i>
<i>EAB</i>	Unknown	a	a b	2	<i>a, b</i>
<i>EAC</i>	7 ^d	h	a	2	<i>-, a</i>
<i>EAD</i>	12	a	a b	2	<i>a, b</i>
<i>EAE</i>	9	a	a b c e f g h i j k l m n o p r s t	17	<i>aeglns, bdgkmpps, defhkmnps, degghkmnps, aeflns, etc</i>
<i>EAF</i>	8	a	a b c d	4	<i>ac, ad, bc, bd</i>
<i>EAG</i>	15	a	a b	2	<i>a, b</i>
<i>EAH</i>	6	h	a b c d e	7	<i>-, a, b, ab, bd, cd, be</i>
<i>EAI</i>	18	c	a b	2	<i>a, b</i>
<i>EAJ</i>	7	c	a b	3	<i>-, a, b</i>
<i>EAK</i>	9	h	a b c d e f g	6	<i>-, acf, acef, ade, adeg, bf</i>
<i>EAL</i>	4	c, d	a b c d f g h i j k l m	6	<i>adhi, bcgi, bdfi, agim, adhjk, adhjl</i>
<i>EAM</i>	11	c, h	a b c d e f g h i j k m	20	<i>-, ab, ade, aem, b, bd, bcdi, cd, etc</i>
<i>EAN</i>	9	c	a b c	3	<i>a, b, bc</i>
<i>EAO</i>	6	d	a b	2	<i>a, b</i>
<i>EAP</i>	Unknown	c	a	2	<i>-, a</i>

^aFrom ref.78.

^ba, Saline agglutination; c, Coombs' test; d, Dextran test; h, hemolytic test.

^cThe absence of detectable factors (null allele, recessive) in any locus is represented by a dash (-).

^dAssignment based on linkage of C and J systems¹.

and limited availability of donors. Blood transfusions in large animals are indicated for certain acute, life threatening conditions and for plasma transfusion when failure of passive transfer occurs. Single, unmatched whole blood transfusions are generally safe and well tolerated. A gross crossmatch is recommended and sufficient when repeated transfusions are required. Matching is done with hemolytic and/or agglutination tests depending of the species and involves testing of the recipient's serum for antibodies against the RBCs of potential donors (major) and of the donors' sera for antibodies against the recipient's RBCs (minor) (see Chapter 139).

Pigs are often used as an animal model for organ or hematopoietic transplantation. Blood group compatibility is an important component of experimental protocols because of problems associated with immune-mediated tissue rejection or the need to provide transfusion support to patients.⁸⁵ In transplant experiments, mismatched transfusions for the *EAA* system result in adverse reactions that include disseminated intravascular coagulation, bleeding, and progressive hypotension^{60,82} rather than typical hemolytic reactions seen in humans.

Horses merit special consideration because whenever a mare is given a whole blood transfusion, she is potentially being sensitized to blood group factors that may subsequently lead to NI problems for her foals. Selection of suitable donors that are negative for highly antigenic factors such as *EAAa* (and without circulating antibodies in the serum) is recommended although currently not practical because very few laboratories can perform the serological test for blood group specificities. Mares that received a blood transfusion should

always be screened for potential risk of NI prior to foaling.

Plasma Transfusion

A blood transfusion may be used to restore fluid loss and replace necessary proteins, and the RBC component may not be essential. In this case, a plasma transfusion may fulfill the clinical requirements. Potential donors can be selected in a hematology laboratory by screening their sera against a RBC panel from 10 to 20 horses to identify those without antibodies with anti-blood group activity. Plasma can be collected and stored frozen to administer as needed.

Neonatal Isoerythrolysis in Horses

Neonatal isoerythrolysis is an acute hemolytic disease of newborn foals caused by immunologically-mediated RBC destruction resulting from maternal-fetal blood group incompatibility. Affected foals are healthy at birth but within 2–5 days develop signs of lethargy, elevated pulse and respiration rates, and clinical evidence of anemia. NI foals are usually from the second or later pregnancies of a mare but, on rare occasions, first foals can be affected. The antibodies that sensitize the RBCs of the foal are passively acquired from the dam's colostrum. Recovery may be spontaneous or the disease may progress to severe anemia and death.

The most common antibodies involved in NI are anti-Aa and anti-Qa, although others are infrequently found. Not all mares negative for *EAAa* or *EAQa* factors become sensitized, although they may have produced Aa-positive or Qa-positive foals. One source of sensi-

zation is whole blood transfusion, but this accounts for only a small number of cases. Mares negative for both Aa and Ca factors may be protected from sensitization to Aa by naturally produced anti-Ca antibodies.¹¹ At present, no simple hypothesis explains why blood group sensitization occurs in only a few percent of mares at risk.

A serum sample taken about 3 weeks before a pregnant mare is due to foal can be screened for evidence of blood group incompatibility. If the test results are positive for hemolytic anti-blood group activity, it is strongly advised to withhold the foal from its dam's colostrum for 36–48 hours before putting it back to its dam's milk. An alternative colostrum and milk source must be provided to the foal during that period.

NI Disease in Mule Foals

Mule breeders are keenly aware that mares bred to jacks (male donkeys) can become sensitized to blood group factors of asses.⁹⁹ Monitoring of a pregnant mare's serum for antibodies against the RBCs of the jack to which she is bred is a prudent precaution to identify mule neonates at risk for NI.

Red Blood Cell Donors for Foals with NI

Appropriate management for a severely anemic NI foal may be a RBC transfusion to alleviate the anemia and prevent death. The best blood donor is one whose RBCs lack the factor to which the mare is making antibodies. The sire of the foal is the worst possible donor since he has the factor to which the mare's immune system has responded. The best immediate RBC donor is the mare. The mare's RBCs should be administered to the foal in a suitable transfusion solution, but first they must be separated from the plasma and washed in saline to remove the antibodies reacting with the foal's RBCs. The mare is unlikely to have a matching blood group type but it provides the foal with vital RBCs that will not be destroyed by the antibodies acquired from the colostrum. Hopefully, in a short time the blood group antibodies will be eliminated from circulation and the foal can make sufficient RBCs of its own to prevent a recurrence of the anemia crisis.

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Granulocyte and Platelet Antigens

JENNIFER S. THOMAS

Granulocyte Antigens

Laboratory Techniques to Identify Neutrophil Antigens and Antigen-Antibody Interactions
Clinical Disorders Associated with Neutrophil Antigens

Platelet Antigens

Laboratory Techniques to Identify Platelet Antigens and Antigen-Antibody Interactions
Clinical Disorders Associated with Platelet Antigens

Acronyms and Abbreviations

EDTA, ethylenediaminetetraacetic acid; GP, glycoprotein; HLA, human leukocyte antigen; HNA, human neutrophil antigen; HPA, human platelet antigen; IMN, immune-mediated neutropenia; IMT, immune-mediated thrombocytopenia; LFA-1, leukocyte function antigen 1; PCR, polymerase chain reaction; PTP, post-transfusion purpura; TRALI, transfusion-related acute lung injury; VWF, von Willebrand factor.

Cell surface antigens on granulocytes and platelets play a role in recognition of self. Antigens vary in their ability to stimulate an immune response and, therefore, in their association with disease. Much of the published research has focused on the role of platelet and granulocyte antigens in immunologic disorders; however, antigen identification is also used as a marker of disease. In veterinary medicine expression of platelet or granulocyte antigens is determined to characterize myeloproliferative disorders. Expression of some antigens, such as P-selectin (CD62P) on platelets, is used to determine cellular activation.²⁹

Immune responses targeting granulocyte or platelet antigens have been implicated in the pathogenesis of primary (autoimmune or idiopathic) and secondary immune-mediated neutropenia (IMN) or thrombocytopenia (IMT), neonatal alloimmune neutropenia or thrombocytopenia, and transfusion reactions.^{9,10,40,41,50} Binding of antibodies to cell antigens may also cause dysfunction of the target cells.^{8,25} Immunologic disorders resembling those listed above have been identified in veterinary species; however, the pathogenesis of these conditions is generally better characterized in humans because transfusion therapy and neonatal cytopenias are more common.⁵⁰ Most of the information provided in this chapter is based upon current understanding of the role of platelet and granulocytes antigens in immunologic disorders in humans. Whenever possible, comparisons between humans and veterinary species will be presented.

Platelet or granulocyte antigens can be specific to one cell type, shared by other blood cells, or widely distributed on systemic cells. Antigens are generally classified based upon their biochemical nature into glycolipids, glycoproteins (GPs), or proteins.^{9,41} The roles of some, but not all, of these antigens in normal cellular function are known. The molecular structures of some of these antigens have been characterized, allowing the development of molecular techniques to detect alterations in the nucleic acid structure of the genes encoding the antigens.

Variations in the structure of an antigen that is present in some, but not all, individuals in a species are termed alloantigens. Alloantigens are the products of polymorphic genes. Most granulocyte and platelet alloantigens have been linked to single-point mutations.²⁷ The prevalence of the different alloantigens in humans varies significantly between ethnic groups.^{8,41} It is likely that similar differences exist in the expression of alloantigens among different breeds and species of animals.⁵⁰

Antibodies directed against alloantigens are termed alloantibodies. Alloantibodies may form when an individual receives a blood product that contains an alloantigen not present on self cells or when there is an incompatibility between fetal and maternal blood cells. Isoantibodies are antibodies that target a nonpolymorphic antigen found on the cells of the vast majority of normal individuals in a species. Generally, isoantibodies form when an individual who has an inherited defi-

ciency of an antigen is transfused with blood products from a normal individual.⁵⁰

Antibodies that are produced against self-antigens are termed autoantibodies. The production of autoantibodies may be idiopathic or may be secondary to an underlying disorder.^{10,55,65} Autoantibodies are sometimes produced following drug administration. In some cases the drug or its metabolite must be present for antibody binding to occur. In other cases the drug induces an antibody that persists after drug administration ceases.³

GRANULOCYTE ANTIGENS

The granulocytes consist of neutrophils, eosinophils and basophils. Neutrophils are present in highest concentrations in the blood and are most commonly implicated in immunologic disorders.⁵⁰ In humans immunologic disorders are associated with antigens in the human leukocyte antigen (HLA) or the human neutrophil antigen (HNA) systems.^{56,57}

Human leukocyte antigens are widespread in distribution and found on many different cell types; HNA antigens are predominantly expressed on neutrophils (Table 93.1). It is currently unclear how many of the HNA antigens are expressed on other leukocytes.⁸ The HNA systems are numbered according to the GP site where they are located. Antigens within each HNA system are listed alphabetically in order of their identification.⁵⁶ The HNA-1 antigens are found on Fc γ RIIIb (CD16b), a low affinity receptor for the Fc portion of immunoglobulins. They vary significantly in degree of glycosylation. The HNA-2 antigen is found on a 56–64kDa GP (CD177) that is involved in adhesion to endothelial cells and subsequent transmigration into the extravascular space. The HNA-3 antigen is located on a 70–95kDa protein whose function is not known. The HNA-4 antigen is located on the CD11b/CD18 complex (Mac-1, CR3), a β_2 -integrin that plays a role in binding to endothelial cells and complement proteins. The HNA-5 antigen is found on the CD11a/CD18 complex (leukocyte function antigen 1 [LFA-1]), a β_2 -integrin involved in adhesion to endothelial cells.^{8,57}

Monoclonal antibodies are used in a variety of veterinary species to identify the GPs present on neutrophils. The relationship between the antigens rec-

ognized by these monoclonal antibodies and the antigens associated with immunologic disorders is not currently known.⁵⁰ Monoclonal antibodies to CD11a (LFA-1) and CD11b (Mac-1; CR3) bind to bovine neutrophils.^{19,54} The monoclonal antibody to CD11a also binds to all other bovine leukocytes but not to erythroid cells or platelets. Monoclonal antibodies to CD11a also recognize antigens on neutrophils, lymphocytes and monocytes from dogs, horses, sheep, goats and rabbits.^{6,28,31,49,59} The antibody to CD11b also binds to bovine monocytes and a subpopulation of B lymphocytes.^{19,54} Antibodies to CD11b bind to neutrophils, monocytes and lymphocytes from sheep, goats, and pigs, as well as neutrophils and monocytes from cats and dogs.^{6,49} Anti-CD16 antibodies bind to canine neutrophils.^{31,59} Additional information on CD antigens is available in Chapter 4.

Laboratory Techniques to Identify Neutrophil Antigens and Antigen-Antibody Interactions

Identification of neutrophil antigens or anti-neutrophil antibodies has traditionally relied on agglutination or immunofluorescence assays.^{56,57} In agglutination assays neutrophils clump if a patient's serum containing autoantibodies is added to neutrophils from a normal individual or if a patient's neutrophils have an alloantigen that is targeted by a specific alloantibody in test serum. In microscopic immunofluorescence assays a fluorescence-conjugated secondary antibody is used to detect binding of a specific alloantibody to its target alloantigen or autoantibodies to target antigens on neutrophils. Genotyping assays using polymerase chain reaction (PCR) techniques are replacing agglutination or immunofluorescence assays in humans to identify many of the major neutrophil antigens.⁵⁷ Similar assays are not available for veterinary species. Monoclonal-antibody capture assays have been developed to detect antibodies to specific HNAs.⁵⁶ Flow cytometry is used to phenotype neutrophils in humans and animals.^{11,49,54,56,57} It is limited by the availability of monoclonal antibodies that specifically target the antigens of interest. Flow cytometry is also used to identify anti-neutrophil antibodies in animals, but specific target antigens have not been identified.⁶² Further discussion of methods to detect anti-neutrophil antibodies is found in Chapter 140.

TABLE 93.1 Human Neutrophil Antigens and Associated Disorders

Antigen Location	CD Designation	Antigen System	Clinical Conditions in People Associated with Antibody-Antigen Interactions ^a
Fc γ RIIIb	CD16b	HNA-1	Neonatal alloimmune neutropenia, primary IMN, TRALI, drug-induced neutropenia
NB1 glycoprotein 70-,96-kDa protein	CD177	HNA-2	Primary IMN, drug-dependent neutropenia, TRALI
		HNA-3	Neonatal alloimmune neutropenia, TRALI, febrile transfusion reaction
MAC-1; CR3	CD11b	HNA-4	Neonatal alloimmune neutropenia, primary IMN
LFA-1	CD11a	HNA-5	Unknown

^aIMN, immune-mediated neutropenia; TRALI, transfusion-related acute lung injury.

CLINICAL DISORDERS ASSOCIATED WITH NEUTROPHIL ANTIGENS

In humans, clinical conditions associated with alloantibody interactions include neonatal immune neutropenia, transfusion-related acute lung injury (TRALI), alloimmune neutropenia after bone marrow transplantation, refractoriness to granulocyte transfusions, and febrile transfusion reactions.^{8,10,57} Febrile transfusion reactions occur when the recipient has alloantibodies directed against antigens present on the donor leukocytes. Febrile transfusion reactions can be minimized by removing leukocytes from RBC and platelet components.⁸ TRALI occurs when alloantibodies to HLA antigens or neutrophil specific antigens cause neutrophils to become entrapped in the pulmonary vasculature.⁵⁷ Binding of alloantibodies to neutrophil antigens also cause functional defects, such as impaired adhesion and altered respiratory burst.^{8,48}

Disorders associated with alloantibody production are not commonly recognized in veterinary species. Neonatal neutropenia is rare but occurs in pigs and foals.^{13,33} It occurs when the dam has been previously exposed to paternal antigens found on the fetal neutrophils and produces alloantibodies that are passed to the fetus in utero or to the neonate following ingestion of colostrum. Febrile reactions in dogs following transfusion of platelet concentrates have been associated with leukocyte contamination.¹ Additional discussion of transfusion reactions is found in Chapters 97 and 100.

In humans autoantibody production is implicated in the pathogenesis of primary IMN, secondary IMN, drug-dependent neutropenia, and autoimmune neutropenia after bone marrow transplantation.⁸ The cause of the autoantibody production is unknown in primary IMN. Studies in humans have implicated antibodies targeting Fc γ RIIIb (anti-HNA-1a and anti-HNA-1g) in many patients.¹⁰ Secondary IMN is associated with systemic immune-mediated disorders, infectious diseases, neoplasia, bone marrow or stem cell transplants, kidney transplants, and drug therapy.¹⁰ The antigenic target in secondary IMN is often unknown. Antibodies bind to antigens on neutrophils and lead to premature neutrophil removal by macrophages. In some cases

autoantibodies target antigens on hematopoietic precursor cells in the bone marrow leading to myeloid hypoplasia. Patients may also have defective neutrophil phagocytosis, as well as concurrent thrombocytopenia or hemolytic anemia. Diagnosis often depends on identification of anti-neutrophil antibodies using agglutination, monoclonal antibody capture assays, immunofluorescence or flow cytometric assays.^{10,21,62}

Immune-mediated neutropenia is infrequently reported in veterinary species.^{7,45} Antibodies generally target antigens on circulating neutrophils, but bone marrow directed immune response causing white cell aplasia has been reported.⁶³ IMN is suspected when an animal has unexplained neutropenia that is responsive to immunosuppressive drugs. Identification of anti-neutrophil antibodies is supportive, but the diagnostic assays required to detect these antibodies are often not performed due to limited availability.⁴⁵ Anti-neutrophil antibodies have been identified in dogs with IMN, but the specific target antigen has not been identified.^{60,62} Drug associated neutropenia is rarely reported. Anti-neutrophil antibodies have been identified in neutropenic dogs receiving an antipsychotic drug or cephalosporin.^{4,35}

Antibodies to neutrophil isoantigens have been identified in human neonates born to mothers who had neutrophils deficient in Fc γ RIIIb.¹⁸ Although not reported, a similar disorder is possible in dogs or cattle with leukocyte adhesion deficiency syndrome, a deficiency of membrane CD11/CD18 complex.⁵⁰

PLATELET ANTIGENS

In humans platelet antigens commonly implicated in immunologic disorders are those in the HLA system and the human platelet antigen (HPA) systems (Table 93.2). HLA antigens are widespread in distribution and found on many different cell types. HPA antigens are found predominantly on platelets, though some are expressed on other blood cells.⁴¹ The HPA antigens are located on membrane GPs and include GP $\alpha_{IIb}\beta_3$ (IIb/IIIa), GPIb/IX/V, GP $\alpha_2\beta_1$ (GPIa/IIa) and CD109.^{14,16,40,41}

TABLE 93.2 Human Platelet Antigen Systems and Associated Disorders

Antigen Location Site	CD Designation	Human Antigen Systems	Clinical Conditions Associated in People with Antibody-Antigen Interactions ^a
GP β_3	CD61	HPA-1, HPA-4, HPA-6, HPA-7, HPA-8, HPA-10, HPA-11, HPA-14, HPA-16w	Neonatal alloimmune thrombocytopenia; PTP; primary IMT
GPIb $_{\alpha}$	CD42b	HPA-2	Primary IMT
GP α_{IIb}	CD41	HPA-3, HPA-9	Neonatal alloimmune thrombocytopenia; PTP; primary IMT
GPIa	CD49b	HPA-5, HPA-13	Neonatal alloimmune thrombocytopenia; PTP; primary IMT
GPIb $_{\beta}$	CD42c	HPA-12	Primary IMT
CD109	CD109	HPA-15	Neonatal alloimmune thrombocytopenia; PTP; refractoriness to transfusion

^aIMT, immune-mediated thrombocytopenia; PTP, post-transfusion purpura.

GP $\alpha_{IIb}\beta_3$ (CD41/CD61) is an integrin that is critical for platelet aggregation. Following activation, GP $\alpha_{IIb}\beta_3$ undergoes a conformational change that allows binding to adhesive proteins such as fibrinogen and von Willebrand factor (VWF). Glanzmann's thrombasthenia, a functional or physical deficiency of GP $\alpha_{IIb}\beta_3$, is found in humans, dogs, and horses. Affected individuals have a bleeding disorder characterized by impaired platelet aggregation.^{5,12} The GP $\alpha_2\beta_1$ complex is a collagen receptor. Alterations in this receptor cause impaired adhesion to collagen and impaired collagen induced aggregation.^{40,42} The GPIb/IX/V complex binds to VWF and plays an important role in platelet adhesion to the vessel wall under high shear-stress conditions.¹⁴ An inherited functional or physical deficiency of GPIb/IX, termed Bernard-Soulier syndrome, is found in humans and is associated with thrombocytopenia, giant platelets, and functional defects.⁴² The role of CD109 is not clear. It is detected on activated platelets and may be involved in cell communication.¹⁶

The HPA alloantigens have been characterized at the molecular level. Most result from a single amino-acid substitution resulting from a single nucleotide change. The frequency of the different alloantigens varies in different ethnic populations.^{40,41}

These major platelet GPs appear to be conserved between the species, though the number of different alloantigens and their role in immunologic disorders in veterinary species is unknown.^{12,34,38} Monoclonal antibodies are used to identify platelet antigens in a number of species. The association between antigens recognized by the monoclonal antibodies and the antigens associated with disease is not known. Monoclonal antibodies against human or porcine CD41 (GP α_{IIb}), CD42a (GPIX) and CD61 (GP β_3) bind to canine and equine platelets.^{2,36,49} Monoclonal antibodies to porcine or ruminant CD41/CD61 (GP $\alpha_{IIb}\beta_3$) bind to bovine, porcine and equine platelets.^{36,43,51} Monoclonal antibodies against human CD61a and CD62P (P-selectin) bind to bovine platelets.⁵⁴ Monoclonal antibodies against human GPIb react with feline platelets.⁵⁸ Canine GPIb α was cloned and is 82% identical with the human sequence in the carboxyl-terminus.²² The nucleotide sequence of canine GP β_3 is 92% homologous with the human sequence.³⁴

Laboratory Techniques to Identify Platelet Antigens and Antigen-Antibody Interactions

Platelets are commonly typed in people for purposes of platelet transfusion. Serologic phenotyping is generally limited to HPA-1a and HPA-5b because of limited availability of reliable alloantibodies to other alloantigens.⁴⁰ Agglutination tests similar to those used to phenotype neutrophils are not reliable for platelets, and platelet phenotyping more commonly utilizes immunoblotting or immunoprecipitation techniques.¹⁶ Flow cytometry is used in human and veterinary medicine but is limited by the availability of reliable monoclonal antibodies.⁴⁰ Genotyping using PCR techniques to identify platelet antigens has become standard practice in human medicine.²⁰

No single test has proven sufficient to detect anti-platelet antibodies in humans.⁵³ Available techniques include platelet immunofluorescence assays, flow cytometry, or monoclonal antibody-specific immobilization of platelet antigen assays. Immunofluorescence, flow cytometry and immunoprecipitation have been used to measure anti-platelet antibodies in veterinary species.^{23,25,30,50} Detection of anti-platelet antibodies is further discussed in Chapters 78 and 140.

Clinical Disorders Associated With Platelet Antigens

Clinical disorders associated with platelet alloantigens are most commonly associated with the ability of alloantigens to induce an immune response; however, polymorphisms in the platelet receptors that occur with the different alloantigens may also cause altered function.⁴⁰ Though conflicting results have been reported, studies suggest that some alloantigens in the HPA-1 system are associated with increased platelet reactivity and increased risk for coronary heart disease and stroke.¹⁴

The binding of autoantibodies and alloantibodies to platelet GPs often causes platelet destruction and thrombocytopenia. These antibodies may also cause platelet inhibition and increased risk for bleeding or platelet activation and increased risk for thrombotic disorders. Functional defects may be present independent of any associated thrombocytopenia.¹⁵ One study suggested that platelets from dogs with IMT have impaired platelet function due to circulating antibodies.²⁶ Platelet aggregation was inhibited in mule foals with neonatal alloimmune thrombocytopenia, possibly due to alloantibody competition with collagen binding sites.⁴⁶

In humans, disorders associated with alloantibody production include neonatal alloimmune thrombocytopenia, post-transfusion purpura (PTP), and refractoriness to platelet transfusions.^{41,47} Neonatal alloimmune thrombocytopenia occurs when the mother produces alloantibodies against a paternal alloantigen expressed on fetal platelets. The majority of human cases are associated with an alloantibody against fetal GP β_3 (HPA-1a). Alloantibodies against fetal GPIa (HPA-5a), CD109 (HPA-15b), or GP α_{IIb} (HPA-3a) are less commonly implicated.⁴⁷ Neonatal alloimmune thrombocytopenia has been described in pigs, horses, and mules.^{17,44,46} The specific target alloantigen was not identified in any of these reports.

Post-transfusion purpura is a rare condition that occurs 5–12 days after an individual receives a blood transfusion. It is most common in HPA-1a negative women who have been previously sensitized by prior transfusion or pregnancy. Other platelet specific antigens or HLA antigens have been less frequently implicated. The immune response causes the destruction of the individual's own platelets, as well as the transfused platelets.⁴¹ PTP has been described in dogs and pigs; however, an association with a specific alloantigen has not been identified.^{32,61}

Platelet refractoriness occurs in humans who receive multiple platelet transfusions. These individuals

produce alloantibodies that result in shortened survival of the transfused platelets. Anti-HLA antibodies are most commonly implicated; however, anti-HPA antibodies are found in a minority of individuals.⁴¹ Platelet refractoriness has been experimentally reproduced in dogs.⁵²

The pathogenesis of IMT is covered further in Chapter 78. Briefly, IMT occurs when antibodies bind to platelet antigens and cause premature destruction of platelets by the mononuclear phagocytic system or lysis in a complement-dependent manner.^{24,65} Recent studies also implicate direct T cell mediated cytotoxicity and impaired megakaryopoiesis and thrombopoiesis in the pathogenesis of the thrombocytopenia.⁵⁵ In humans, the target antigens are most commonly located on GP $\alpha_{IIb}\beta_3$.⁶⁵ Other targets include specific regions on GPIb/IX, GPIV, and GP $\alpha_2\beta_1$.²⁴ Antibodies to multiple antigens are found in many patients.⁵⁵

Cryptic epitopes, particularly involving GP $\alpha_{IIb}\beta_3$, have been implicated in both initiation and perpetuation of thrombocytopenia in humans with IMT.⁶⁵ These epitopes are normally hidden but may become exposed and initiate an immune reaction following an insult, such as an infection. Exposure of cryptic antigens is also involved in EDTA-dependent pseudothrombocytopenia in humans. EDTA binds calcium which causes a conformational change in GP $\alpha_{IIb}\beta_3$, exposing normally hidden epitopes which bind to circulating antibodies. EDTA-dependent pseudothrombocytopenia has been rarely reported in dogs, horses and pigs. The underlying mechanism in these veterinary species has yet to be determined.⁶⁴

Immune-mediated thrombocytopenia is commonly diagnosed in dogs and sporadically identified in other species. In primary IMT the immune response is directed against autoantigens and an underlying disease is not identified. In secondary IMT the immune response is associated with an underlying disorder (e.g. systemic immune-mediated disease, infection, neoplasia). The antibodies can be directed against either autoantigens, foreign antigens absorbed to platelet membranes, or altered platelet antigens. Platelet surface associated antibodies have been demonstrated in dogs, cats, and horses with IMT.^{23,25,30,39} In most veterinary cases the target antigens on the platelet membrane are not identified. GP $\alpha_{IIb}\beta_3$ and GPIb were identified as target antigens in some dogs with a clinical diagnosis of primary IMT.³⁰

Isoantibodies have been detected in humans with an inherited deficiency of platelet membrane antigens. Humans with Glanzmann's thrombasthenia produce isoantibodies following transfusion of platelets from normal individuals.³⁷ The isoantibodies appear to target epitopes on β_3 or intact $\alpha_{IIb}\beta_3$, leading to inhibited function and rapid removal of the transfused platelets. Although not reported, similar responses could occur in animals with inherited membrane GP deficiencies.⁵⁰

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Principles of Canine and Feline Blood Collection, Processing, and Storage

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Storage

Red Blood Cell Products

Plasma Products

Platelet Products

Quality Control

Acronyms and Abbreviations

ACD, acid citrate dextrose; APS, anticoagulant-preservative solution; AS, additive solution; CPD, citrate-phosphate-dextrose; CP2D, citrate-phosphate(2)-dextrose; CPDA-1, citrate-phosphate-dextrose-adenine-1; EBV, estimated blood volume; FFP, fresh-frozen plasma; FP, frozen plasma; FWB, fresh whole blood; Hgb, hemoglobin; MAP, mean arterial pressure; PCV, packed cell volume; pRBCs, packed red blood cells; PC, platelet concentrate; PRP, platelet-rich plasma; RBC, red blood cell; RT, room temperature; WB, whole blood.

BLOOD BANKING – INTRODUCTION

The advent of large-scale veterinary blood banks has made the practice of high-quality transfusion medicine possible by increasing availability of blood components. Strict adherence to blood collection, processing, and storage guidelines is essential to providing safe and effective transfusions. This chapter details the currently accepted blend of human protocol and veterinary modifications where necessary for the collection, processing, and storage of canine and feline blood components.

DONOR CONSIDERATIONS

Canine

Veterinary blood banks currently use resident dogs, client-owned dogs, or a combination of the two to meet supply demands. Because human blood packs are used

to collect blood, a standard donation is one unit of blood (450 ± 45 mL). Based on an estimated blood volume (EBV) of 85 mL/kg body weight¹⁵ and a recommended collection volume of 15–22% EBV,¹¹ the maximum donation volume is approximately 19 mL/kg. In a study evaluating changes in systolic blood pressure in 19 Greyhounds (27–41 kg) after donating 450 mL blood, the mean systolic pressure dropped from 145 to 134 mmHg, not a clinically relevant decrease.¹¹ Recommended minimal donor body weight varies among veterinary blood banks from 23 to 27 kg, with dogs of this size able to donate a unit as often as every 3–4 weeks without need for nutritional supplementation.²¹ Donor blood-type issues are discussed in Chapters 92 and 100.

For the safety of both the blood donor and recipient, the health of a dog is evaluated prior to enrollment as a donor and then annually through a complete medical history, physical examination, and laboratory evaluation including a hemogram, serum biochemistry profile,

and infectious disease screening. In addition, a brief history, physical examination, and measurement of packed cell volume (PCV) or hemoglobin (Hgb) concentration is performed prior to each blood donation; donor PCV or Hgb concentration should be at least 40% or 13 g/dL, respectively. Donors should be current on vaccinations and maintained on heartworm prevention and flea and tick control. Most other drugs are unacceptable for use during the donation period because of unknown effects on blood quality and potential for adverse reactions in the recipient.

Minimal screening for infectious diseases based on the American College of Veterinary Internal Medicine Consensus Statement is recommended if there is risk for exposure.³⁰ These diseases include *Babesia canis* and *B. gibsoni*, *Leishmania* spp., *Ehrlichia canis* and other *Ehrlichia* spp., *Anaplasma* or *Neorickettsia* spp., and *Brucella canis*. Donors should be excluded based on a positive serologic test for a vector-borne disease. Serologic negative donors may be further screened by polymerase chain reaction assays. Screening for *Trypanosoma cruzi*, *Bartonella vinsonii* and hemotropic *Mycoplasma* spp. may also be considered. Exclusion from a blood donor program does not imply that the animal should be treated for the disease.

Prior transfusion is considered an exclusion criterion for dogs to become blood donors. Following a blood transfusion dogs may develop antibodies against foreign red blood cell (RBC) antigens, resulting in an increased risk of incompatibility reactions to their plasma. Previous pregnancy has long been considered an exclusion criterion in the event that a dog negative for a specific RBC antigen becomes sensitized by her pups positive for that antigen. This risk should be minimal considering the zonal placentation of dogs, and there is little evidence to support this exclusion criterion.⁵

Feline

Because cats typically require sedation for blood collection, most blood banks rely on resident cats. The estimated blood volume of a cat varies according to the reference, ranging from 40 to 60 mL/kg in the anesthetized cat¹² to as high as 67 mL/kg.²⁴ Donation volume should be calculated based on lean body weight; recommendations for *maximum* donation range from 11 to 15 mL/kg, and standard volumes collected (including anticoagulant) range from 50 to 70 mL.^{1,13,14} In one study of 26 cats (5–8 kg), blood pressure was measured before and after donation of a 50 mL unit using sevoflurane anesthesia; mean arterial pressure (MAP) decreased from 87 mmHg (range 59–127 mmHg) to 71 mmHg (range 44–116 mmHg).¹⁴ In another study with eight cats (4.0–6.5 kg) sedated with butorphanol, acepromazine, ketamine and diazepam, MAP dropped from 108 mmHg (range 80–140 mmHg) to 53 mmHg (range 20–100 mmHg) after collection of a 50 mL unit.⁴ No cats in either study showed effects of hypotension upon recovery. However, in the second study, when the same volume was drawn in awake or mildly sedated cats

through vascular access ports, two cats exhibited transient distress, including vocalization, vomiting, and defecation.⁴ This is in contrast to another study of four awake cats using vascular access ports where no signs of hypotension were noted with donations of 10 mL/kg.²⁰ In the authors' experience cats donating less than 11 mL/kg are less likely to have hypotension-related problems. Most blood banks give donors supplemental iron. Assuming a similar mean concentration of 0.5 mg iron/mL blood in cats as in humans, a 60 mL donation represents a loss of 30 mg of iron. This may be replaced with 150 mg oral ferrous sulfate, typically divided into several doses in between donations and adjusted based on pre-donation PCV.

Initial and pre-donation health assessment and health maintenance are similar to canine donors. Donor PCV or Hgb concentration should be at least 30% or 10 g/dL, respectively. Feline donors should be kept indoors to minimize the risk of infectious disease transmission. Cats testing positive for feline leukemia virus and feline immunodeficiency virus by enzyme-linked immunosorbent assay and for hemotropic *Mycoplasma* spp. by cytology or polymerase chain reaction test should not be used as donors.³⁰ Screening for *Cytauxzoon felis* and rickettsial diseases may be considered based on exposure risk. As with dogs, exclusion from a blood donor program does not imply that the cat should be treated for the disease. In addition, one must consider the potential ramifications of false-positive results for feline viral infections, namely unwarranted euthanasia.

BLOOD COLLECTION PRINCIPLES

Blood is collected as whole blood (WB) and then separated into components by centrifugation. Alternatively, single components may be collected by apheresis. For optimal quality, WB is collected into commercially available, sterile, airtight systems that allow for subsequent processing and storage of components without exposure to the environment. These systems, known as closed systems, prevent exposure of blood components to air except when the component unit is entered with an administration set for transfusion. Conversely, an open system has one or more additional sites of entry and possible bacterial contamination, such as occurs when preparing for blood collection into syringes, acid-citrate-dextrose (ACD) bottles, or bags to which anticoagulant is added after manufacture. Components prepared from blood collected into an open system are not intended for storage. If an open collection system is used, the American Association of Blood Banks and Council of Europe advise that RBC products (stored at 1–6 °C) should be used within 24 hours and platelet products (stored at 20–24 °C) should be used within 4 hours.^{6,10} The shelf-life of plasma from an open system is unaffected if it is frozen within 8 hours.^{6,10} Throughout the following sections, processing and storage will refer to closed systems unless otherwise specified. Detailed illustrated methods of blood collection and processing

have been previously described and will be summarized here.^{1,19,23}

BLOOD COLLECTION SUPPLIES

Canine

Commercial 450 mL collection bags manufactured for human blood banking are used for canine donations. These contain an anticoagulant-preservative solution (APS) and a sterile collection line with a 16-gauge thin-walled needle attached to the bag. The bags are available in a variety of configurations consisting of a primary collection bag with attached satellite bags (0 to 4). These satellite bags, some of which may contain RBC nutrient additive solutions (AS), are used for the separation of components as discussed later. After centrifugation and removal of plasma from the main collection bag, the nutrient AS is added to the packed red blood cells (pRBCs). Various AS are composed of saline, adenine, and dextrose ± mannitol ± citrate-citric acid, and are intended to extend the shelf-life of stored pRBCs. There are several APS and AS available that allow storage of canine pRBCs for approximately 1 month (Table 94.1).

Collection bags are available in a variety of sizes (150 mL, 250 mL, 350 mL, and 500 mL) and contain the appropriate amount of APS and AS. Another option for collection of <450 mL WB is to transfer the unwanted portion of the APS from the primary bag of a 450 mL pack into an empty satellite bag, which is later discarded. This allows collection of proportionately smaller units in a closed system without excessive concentration of anticoagulant; the desired ratio of APS to blood is 1.4:10.⁶

Feline

Closed collection systems designed specifically for feline blood donation are not currently commercially available. However, a feline closed collection system has been prepared by sterilely docking an 18-gauge

apheresis needle (SysLoc Safety A.V. Fistula Needle Set, JMS, Singapore) to a 75 mL pediatric transfer pack (Pedi-Pack, Genesis BPS, Hackensack, NJ) containing APS using a commercial sterile tubing welder (Terumo, Elkton, MD).²⁵

Current open collection systems for cats involve collecting blood by jugular venipuncture using a 19-gauge butterfly needle attached to a syringe or collection bag. The systems are open because APS must be transferred using a syringe and needle from a multi-use container to the collecting syringe or bag. The APS used most often is citrate-phosphate-dextrose-adenine-1 (CPDA-1) at an APS: blood ratio of 1:7 (e.g. 7.5 mL CPDA-1 + 52.5 mL collected blood for a final volume of 60 mL). Open systems include: (1) collection into syringes for prompt transfusion of WB; (2) commercial 100 mL bags and collection tubing with attached butterfly needle for gravity collection; (3) attached three-way stopcock, 60-mL syringe and butterfly needle for syringe collection and subsequent transfer to bag (Animal Blood Resources International, Stockbridge, MI); and (4) collection into syringes (preferably 35 or 60 mL) and then careful injection (to avoid hemolysis) into a transfer pack, either 75 mL (Pedi-Pak), or 150 mL (Fenwal). If an open system for cats is used, care must be taken to maintain sterility where possible, especially if the blood or blood components are to be stored. Periodic culture of stored bags is also recommended as a quality control procedure.

Apheresis

During apheresis WB is fractionated and unwanted components are returned to the donor, permitting collection of a greater quantity of the desired component. Apheresis may be performed as an extension of WB donation, with repetitive donations, separation, and return of unwanted components to the donor. However, the use of automated processors that perform continuous and discontinuous flow centrifugation apheresis is preferred due to greater efficiency and improved quality of the blood component. Apheresis has been used

TABLE 94.1 Anticoagulant-Preservative Solutions and Red Blood Cell Nutrient Additive Solutions in Commercially Available Blood Collection Packs and Associated Shelf-Life of Canine Packed Red Blood Cells

Collection Bag APS ^a	Satellite Bag AS ^b	Shelf-Life of Canine pRBCs Stored at 1–6 °C ^c (days)
Acid-citrate-dextrose (ACD)	None	Not reported
Citrate-phosphate-dextrose-adenine-1 (CPDA-1)	None	20 ^d
Citrate-phosphate-dextrose (CPD)	AS-1 (Adsol, Fenwal, Deerfield, IL)	37 ^e
	AS-5 (Optisol, Terumo, Somerset, NJ)	35 ^f
Citrate-phosphate-2-dextrose (CP2D)	AS-3 (Nutricel, Medsep, Covena, CA)	35 ^g

^aAPS, anticoagulant-preservative solution.

^bAS, additive solution.

^cpRBCs, packed red blood cells.

^dRef. 22.

^eRef. 29.

^fShelf-life based on clinical experiences at Ontario Veterinary College and Washington State University College of Veterinary Medicine.

^gRef. 32.

mostly for the collection of platelets in dogs and is discussed in Chapter 97.

PREPARATION AND COLLECTION OF WHOLE BLOOD

Canine

A full unit contains 450 mL of WB, but 10% variation (405–495 mL) is acceptable considering the volume of anticoagulant in the collection bag. The volume of collected blood is estimated by weight. The specific gravity of whole blood at 37°C is 1.053, so an ideal unit weighs 474 g and the acceptable range is 426–521 g.^{6,28} The minimum acceptable under-draw for blood to be used to prepare pRBCs is 300 mL (316 g), in which case the plasma is discarded as it contains excessive anticoagulant. Weight during collection may be determined by placing the bag on a scale or by using a rocker which automatically clamps the collection tube at the desired weight (e.g. Sebra, Tucson, AZ; Docon, MacoPharma, France).

The jugular vein is the only recommended site for routine blood donation. The authors' choice of position for the donor is lateral recumbency on a table, but sternal recumbency or sitting on a table or floor may be better in some circumstances. The venipuncture site is clipped and surgically prepared.

Blood may be drawn into collection bags aided only by gravity and donor blood pressure, in which case the bag should be continuously and gently rocked by an assistant during collection, which typically takes 5–15 minutes. Alternatively, light suction may be used, shortening collection time to 3–10 minutes. The blood collection bag is hung from a hook inside a clear cylinder with a flat lid (Animal Blood Resources International) such that the blood will be forced to pass through the APS. The collection line with attached needle is brought through a notch between the cylinder and lid, and a tube clamp is placed on the line near the needle. A vacuum source is connected via tubing to an inlet in the chamber. The cylinder is placed on a scale, the scale is tared to zero, and the suction is adjusted to –127 to –178 mmHg (–5 to –7 inches Hg).¹⁶ With one hand, the phlebotomist puts gentle pressure on the jugular vein below the prepared skin site, and the needle held in the other hand is inserted through the prepared site into the donor's jugular vein in either direction. When the tubing clamp is removed, blood flows into the collection line to the bag as the scale measures the grams of blood collected. When the desired amount has been obtained, pressure is released from the jugular below the venipuncture site, the line is clamped, and the needle is removed from the vein as pressure is applied over the site.

During blood collection, the well-being of the donor should be constantly monitored. Mucous membrane color, pulse rate and strength, respiratory rate, and donor attitude are easily evaluated by the phlebotomist and assistant. Any indication of donor compromise is cause for discontinuation of the collection.

Once the needle is removed from the donor, the blood in the tubing is stripped into the bag using a tube stripper, and the bag is gently rocked to ensuring adequate mixing. The bag is then manually compressed to refill the tubing, which is sealed (using hand sealer clips or a thermal sealer) at several points to provide small segments containing WB for quality and compatibility testing.

Feline

Sedation/anesthesia is usually achieved using ketamine and midazolam (ketamine 10 mg/kg + midazolam 0.2 mg/kg IM, or ketamine 2 mg/kg + midazolam 0.1 mg/kg IV) or sevoflurane by facemask.²⁷ Pre-medication may be given with butorphanol (0.4 mg/kg IM). The cat is positioned in lateral (preferred by authors due to steadiness) or sternal recumbency, and the jugular venipuncture site is prepared as for the dog. If a syringe or vacuum chamber is used, only gentle suction (–76 mmHg [–3 inches Hg]) is applied to avoid collapsing the vein or causing hemolysis. Blood collection is typically completed within 3–5 minutes. Careful monitoring of the cat is essential because hypotension may be encountered despite all precautions, and oscillometric monitoring should be considered. Some blood banks routinely give donors intravenous and/or subcutaneous saline at a total dose of 2–3 times the volume of blood drawn, while others only treat the donors if hypotension or signs of hypotension occur. However, collection of smaller volume units (≤50 mL) to reduce the risk of hypotension is safer for the donor and may obviate the need to supplement with fluids. Vascular access ports have been evaluated for donation to avoid anesthesia.^{4,20}

SEPARATION OF WHOLE BLOOD INTO COMPONENTS

A flowchart for the separation of fresh whole blood (FWB, less than 8 hours old) by centrifugation is presented in Figure 94.1. Separation requires a large capacity, floor-model, swing-bucket, temperature-controlled centrifuge.

Canine Packed Red Blood Cells and Plasma

Standard separation of canine FWB into pRBCs and fresh plasma is begun by centrifugation of the blood bags at 5000 × g for 5 minutes at 4°C. The centrifugation time includes acceleration time but not deceleration time. To transfer the plasma to a satellite bag, the primary bag is placed in a plasma extractor, and the seal in the tubing of the primary bag is broken. The plasma extractor is slowly engaged and allowed to gently squeeze the primary bag, which expresses the plasma into the satellite bag. Plasma may be transferred entirely to one satellite bag or divided between two or three bags. Expression of plasma is stopped by clamping the tube. If the pRBCs are to be stored in CPDA-1 without

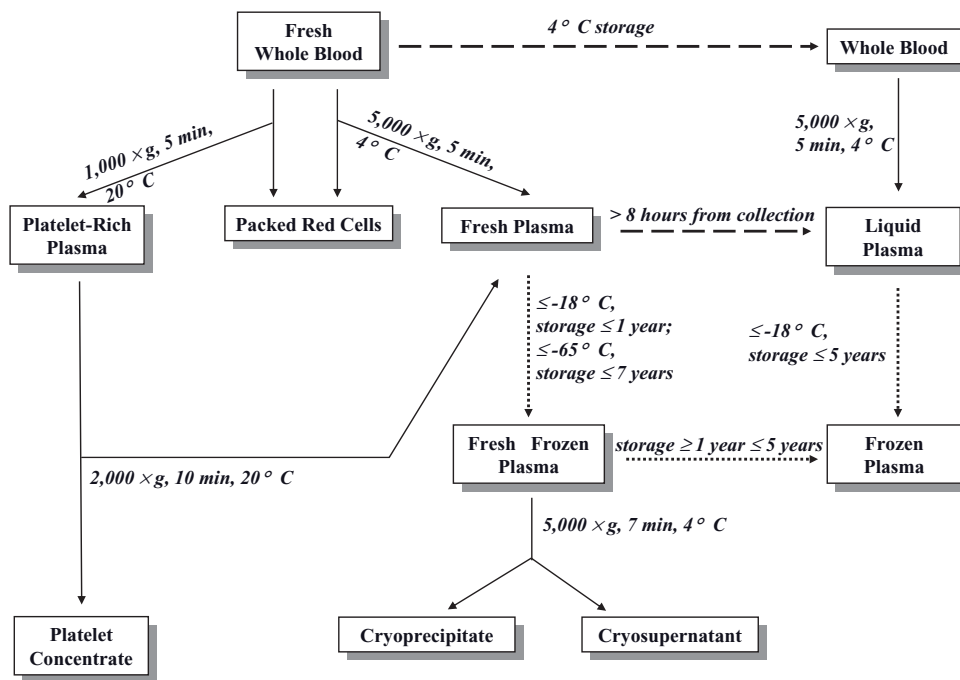


FIGURE 94.1 Flowchart for separation of canine fresh whole blood into components. (Adapted from Abrams-Ogg ACG. Practical blood transfusion. In: Day M, Mackin A, Littlewood J, eds. BSAVA Manual of Canine and Feline Haematology and Transfusion Medicine. Gloucester: British Small Animal Veterinary Association. 2000;263–303, Fig. 15.19, with permission.)

an AS, the pRBCs must have a PCV $\leq 80\%$. This requires leaving ≥ 50 mL of plasma with the pRBCs, which may be accomplished by stopping the expression of plasma when the buffy coat/plasma interface is about 2 cm from the top of the bag. If the pRBCs are to be stored in citrate-phosphate-dextrose (CPD) or CP2D (which contains twice as much dextrose as CPD) with an AS, all of the plasma may be removed from the pRBCs, as the AS will provide the pRBCs with the necessary fluid and nutrients. Once the plasma has been removed to one or more satellite bags, the AS is added to the pRBCs. The seal in the tubing to the AS bag is broken, and the solution is transferred into the primary bag by gravity and gently mixed. If desired, half of the pRBCs can then be transferred to an additional empty satellite bag if available to prepare half-units. The tubing attached to each component bag is then sealed.

Each bag of component is weighed and the weight of the component calculated by subtracting the weight of the bag. Volumes of plasma and pRBCs at room temperature (RT) are then estimated by dividing by 1.026 and 1.056, respectively.²⁸ (These values are for humans, and specific gravity varies with temperature, PCV, and protein concentration, but effects on volume estimates are clinically negligible.) Each component bag is labeled as to product, blood type, donor, volume, and dates of collection and expiration.

Fresh plasma contains albumin, globulins, and maximum possible quantities of all coagulation factors. If this plasma is stored below -18°C within 8 hours of collection, it is labeled fresh-frozen plasma (FFP). If the plasma is not placed in a freezer within 8 hours of collection, it may still be frozen and labeled frozen plasma (FP). There are negligible differences between FFP and FP with respect to albumin, globulins and α -

macroglobulins, vitamin-K dependent factors, and anti-thrombin.⁶ Plasma products are discussed further in Chapter 96.

Canine Cryoprecipitate and Cryosupernatant

Once frozen, FFP may be further processed into cryoprecipitate and cryosupernatant, also referred to as cryopoor plasma and cryoprecipitate-reduced plasma. When separating plasma from FWB with the intention of further processing into cryoprecipitate, the plasma is transferred into a satellite bag and another empty satellite bag is left attached via tubing. The tubing connecting the two is temporarily obstructed by folding and securing with a rubber band. The two bags are frozen together in a cardboard plasma box. After freezing, the unit is slowly thawed in a refrigerator at $1\text{--}6^{\circ}\text{C}$. When the unit reaches a slushy consistency, it is centrifuged at $5000 \times g$ for 7 minutes and all but 10–15 mL of the supernatant plasma (cryosupernatant) is transferred to the empty bag, leaving the precipitate (cryoprecipitate) and small amount of plasma in the first bag. The bags are separated, sealed, labeled and immediately refrozen. Cryoprecipitate contains von Willebrand factor, factor VIII, fibrinogen, fibronectin, and factor XIII and is primarily indicated in the management of bleeding in dogs with von Willebrand disease and hemophilia A (see Chapter 96).

Canine Platelet-rich Plasma and Platelet Concentrate

Fresh whole blood can also be separated into platelet-rich plasma (PRP) and pRBCs. The FWB may be

allowed to rest for 1 hour after collection to minimize platelet activation, and the bag should be gently massaged before centrifugation to re-suspend platelets.¹⁷ Centrifuge temperature should be set at 20°C. Centrifugation to make PRP uses lighter gravitational forces than for standard separation of pRBCs and plasma, and isolates the platelets in the plasma above the buffy coat. When the PRP is expressed into the satellite bag, expression should be stopped when the RBC-plasma interface is 1 cm from the top of the bag to minimize leukocyte and RBC content of PRP while not sacrificing platelet yield.⁷ One unit of FWB (450 ± 45 mL) yields 1 unit of PRP.

Percent platelet yield (platelets in PRP/platelets in FWB × 100) and PRP platelet count vary with the donor, donor platelet count, blood volume being centrifuged, technician, centrifuge, and centrifugation protocol. In a study comparing protocols to make PRP from small volumes of canine blood, protocols with shorter centrifugation times and higher gravitational forces had better yields than protocols with longer times and lower forces.⁸ Most current blood bank protocols use about 1000 × *g* for 4–6 minutes or 2000 × *g* to 2500 × *g* for 2.5–3 minutes.² The brake on the centrifuge should be turned to a low setting or turned off. In the protocol used by the authors (1000 × *g* for 4 minutes), average yield is about 80% (range 35–97%), resulting in a mean of 6 × 10¹⁰ platelets/unit (range 3 × 10¹⁰ to 10 × 10¹⁰ platelets/unit).^{2,3} A second centrifugation of the FWB can be used to increase yield, and is particularly useful with greyhounds which have low normal platelet counts (W.J. Dodds, personal communication).

Platelet concentrate (PC) may be prepared by centrifugation (2000 × *g* for 10 minutes) of PRP to pellet the platelets. The platelet-poor plasma (PPP) is expressed, leaving 35–70 mL of plasma and the platelet pellet behind in the satellite bag. The resulting PC is left undisturbed for 60–90 minutes to promote disaggregation. Gentle manual kneading and agitation are then used to disperse macroscopic leukocyte-platelet aggregates and re-suspend the platelets. The PPP may be used as fresh plasma or FFP. One unit of PRP yields 1 unit of PC. Units of PC can be pooled via transfer tubing. The pooled units are centrifuged at 570 × *g* for 15 minutes and PPP is expressed, leaving behind 8–10 mL per unit pooled. Once pooled, the concentrate needs to rest for at least 15 minutes at room temperature, prior to resuspension (K.J. Wardrop, personal communication).

Feline Blood Components

Feline FWB is placed in one bag, and the tubing between this bag and an empty satellite bag is obstructed by folding and securing with a rubber band or a hand sealer clamp. Due to the relatively small volume of blood being separated, the collection/satellite bags may be bound (via rubber band or tape) to a 500 mL bag of saline to prevent the bags from collapsing during centrifugation. The blood bags are centrifuged at 3000 × *g* for 10 minutes at 4°C, and separation is continued as

for dogs. The pRBCs may be stored without addition of AS (in which case 10 mL 0.9% sodium chloride is added just prior to administration to the patient), or 10 mL AS may be added to pRBCs prior to storage.

Feline PRP is rarely prepared due to a limited clinical need, as well as technical challenges; FWB is more likely to be administered to thrombocytopenic or thrombopathic cats with severe bleeding. However, preparation of feline PRP has been described, with the transfused platelets exhibiting *in vivo* efficacy.^{8,9}

STORAGE

Red Blood Cell Products

Units of WB and pRBCs are stored at 1–6°C. A refrigerator with an alarm to indicate unsafe temperatures is ideal. Alternatives include a regularly-checked thermometer placed in a container of fluid the volume of a small unit and HemoTemp II blood bag thermometer labels (Biosynergy, Elk Village, IL).

Red blood cell viability and function decrease during storage as a result of physical and metabolic changes collectively known as the *storage lesion* (see Chapter 95). Shelf-life is defined as the number of days after collection, assuming proper closed system collection and storage, at which 75% RBC viability is maintained.^{6,10} Viability is measured as 24-hour post transfusion survival of radiolabeled or biotinylated RBCs.^{29,31,32} Shelf-lives for canine pRBCs prepared in closed systems are summarized in Table 94.1.

Plasma Products

All plasma products should be stored at –18°C or lower in a cardboard plasma box which protects the bag from breakage. A freezer with an alarm to indicate unsafe temperatures is ideal. The shelf-life of FFP is 1 year from collection if frozen at –18°C (some countries specify –30°C) and 7 years if frozen at or below –65°C.⁶ If a regular household freezer is used, exposure of the FFP to repeated freeze/thaw cycles will result in loss of the more labile plasma coagulation factors. Expired –18°C stored FFP from dogs and cats may be relabeled as FP and stored for an additional 4 years.¹⁹ Expired FFP is used primarily for treatment of hypoproteinemia and vitamin-K antagonist poisoning. The shelf-life of cryoprecipitate and cryosupernatant is 1 year from the date of collection, regardless of when during that year it was made from FFP.^{6,19}

Platelet Products

Canine PRP and PC are most often produced on an as-needed basis. Current guidelines for human PC allow RT storage for 5–7 days, although there is increased risk for bacterial proliferation at RT storage.²⁶ The PCs are stored in plastic bags under continuous gentle agitation. The composition of the plastic bag, high surface area: volume ratio, and agitation facilitate gas exchange

and sustain aerobic metabolism, and thereby platelet viability. Canine PRP-derived PCs stored for 7 days at RT with to-and-fro agitation in polyolefin bags maintained platelet numbers, metabolic activity, and pH above 6.0, although there was progressive reduction in in vitro aggregation.³

QUALITY CONTROL

Units of pRBCs should be visually inspected during storage and prior to administration. The presence of murky, purple, brown, or red supernatant, RBC mass that appears purple in color, visible clots, or differences in color between the unit and the tubing segments gives reason to suspect bacterial contamination.^{13,18}

Similarly, units of FFP should be inspected when issued for transfusion and discarded if cracks or leaks are noted.

Inadvertent thawing during storage may significantly reduce levels of factor VIII and von Willebrand factor, and allow for bacterial proliferation. In the absence of a freezer alarm or chart recorder, the following techniques may be used to detect inadvertent thawing and refreezing of a unit after its original freezing: (1) Store the plasma box initially laying flat in the freezer. After complete freezing, shift the box to sit on one end. Air bubbles present in the bag will move from the front edge to the upper edge of the bag if thawing occurs, and the unit will also be thicker on the lower edge. (2) Place a rubber band around the unit before freezing, so that it makes an indentation in the bag. After complete freezing, cut the rubber band. If thawing occurs, the indentation left by the rubber band will be less obvious or will vanish.

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Red Blood Cell Transfusion in the Dog and Cat

MARY BETH CALLAN

Indications

- Transfusion Threshold
- Whole Blood versus Packed RBCs
- Fresh versus Stored RBCs
- Additional Benefits of RBC Transfusions
- Administration
 - Warming of Blood

- Blood Filters and Infusion Devices
- Route, Volume, and Rate of Transfusion
- Massive Transfusion
- Patient Monitoring

Acronyms and Abbreviations

BT, bleeding time; 2,3-DPG, 2,3-diphosphoglycerate; FFP, fresh frozen plasma; FWB, fresh whole blood; Hct, hematocrit; Hgb, hemoglobin; [Hgb], hemoglobin concentration; PCV, packed cell volume; pRBCs, packed red blood cells; RBC, red blood cell; SWB, stored whole blood; WB, whole blood; WBC, white blood cell.

The term red blood cell (RBC) transfusion includes administration of packed red blood cells (pRBCs), fresh whole blood (FWB), or stored whole blood (SWB). With a growing knowledge and expertise in veterinary transfusion medicine, as well as establishment of more commercial animal blood banks, the administration of pRBCs rather than whole blood (WB) has become more commonplace for treatment of anemia in dogs^{4,11,24} and, more recently, cats.^{5,12,17} Blood component therapy addresses a patient's specific transfusion needs while maximizing the use of a blood unit and decreasing potential adverse events associated with WB transfusion.

INDICATIONS

Red blood cell transfusions are indicated in the treatment of anemia caused by hemorrhage, hemolysis, or ineffective erythropoiesis. Because oxygen is poorly soluble in plasma, nearly all oxygen contained in blood is carried by hemoglobin (Hgb). Therefore, RBC transfusions increase the oxygen-carrying capacity of the anemic patient and thereby treat or prevent inadequate delivery of oxygen to tissues, with consequent tissue hypoxia. Hemorrhage (acute or chronic) was the major cause of anemia in 70% of dogs receiving RBC transfusions.^{4,11} Blood loss anemia was also the most common indication (44–52%) for RBC transfusions in cats, followed by ineffective erythropoiesis (38%).^{12,26}

Transfusion Threshold

The decision to administer a RBC transfusion is usually based on a measurement of the patient's packed cell volume (PCV), hematocrit (Hct), or Hgb concentration ([Hgb]) and, more importantly, on clinical evaluation of the patient. A "transfusion trigger" or threshold PCV below which a RBC transfusion is administered, has not been clearly defined in human or veterinary medicine. While for many years the standard transfusion threshold for a normovolemic anemic human patient was an Hct of 30% and [Hgb] of 10 g/dL, a more recent trend has been to lower this threshold.¹³ In an analysis of 45 observational studies that assessed the independent effect of RBC transfusion on human patient outcomes, the risks of transfusion outweighed the benefits in 42 studies.¹⁵ Acknowledging the inherent limitation in their analysis of cohort studies, the authors concluded that in adult, intensive care unit, trauma, and surgical patients, RBC transfusions are associated with increased morbidity and mortality and recommended an [Hgb] < 7 g/dL as a transfusion threshold for hemodynamically stable patients.¹⁵ In a landmark multicenter, randomized, controlled clinical trial, the Transfusion Requirement in Critical Care (TRICC), "restrictive" and "liberal" RBC transfusion strategies in the management of 838 critically ill patients with [Hgb] < 9 g/dL were compared.⁷ The "restrictive" group received blood when the [Hgb] fell below 7 g/dL to maintain the [Hgb] at 7–9 g/dL, whereas the "liberal" group received blood

to maintain the [Hgb] at 10–12 g/dL. Overall, the 30-day mortality was similar in both groups, but the mortality rate during hospitalization was significantly lower in the “restrictive” group, suggesting that a restrictive strategy of RBC transfusion is at least as effective as and possibly superior to a liberal transfusion strategy in critically ill patients.⁷ In a recent review of RBC transfusion in human clinical practice, the following generalizations were made: (1) the physiology of oxygen delivery and clinical data indicate little need to transfuse patients with [Hgb] \geq 10 g/dL; (2) at [Hgb] = 8–10 g/dL, the risk of hypoxic organ damage is low for most patients; and (3) patients with [Hgb] $<$ 6 g/dL are usually at substantial risk, particularly if ongoing bleeding is a possibility.¹³

Currently, there are no clinical studies evaluating RBC transfusion threshold (or increased morbidity and mortality associated with RBC transfusion) in dogs and cats. However, it is well recognized that measurement of Hct, [Hgb], or PCV alone is an inadequate determinant of the threshold for RBC transfusion because many additional factors (e.g. cardiac output and oxygen consumption) are involved in adequacy of tissue oxygenation. Also, in patients with hypovolemic anemia, the PCV is falsely elevated: when the total blood volume normalizes because of an increase in the plasma volume, the PCV decreases. Animals with chronic anemia (e.g. anemia of chronic renal failure) typically better cope with a lower PCV than do animals with an acute onset of anemia (e.g. an acute blood loss or hemolytic crisis) because of cardiovascular and other compensatory mechanisms. Because many factors can influence the need for a RBC transfusion, it is imperative that clinical judgment, not PCV, be the ultimate factor in the decision to administer RBCs to a patient. Tachycardia, poor pulse quality, pallor, lethargy, weakness, and decreased appetite are important clinical signs and symptoms that may indicate that a patient may be in need of additional oxygen-carrying support.

Whole Blood Versus Packed RBCs

The majority of canine and feline blood collected at commercial blood banks and veterinary teaching hospitals is processed into pRBCs and fresh frozen plasma (FFP) (see Chapter 94).

Administration of pRBCs is appropriate in the medical management of anemia resulting from any cause, but whole blood (WB) transfusion may be considered in certain situations. By definition, FWB is blood that is less than 8 hours old from the time of collection and has not been refrigerated; therefore, FWB contains functional platelets, coagulation factors, and plasma proteins in addition to RBCs. Potential indications for FWB include anemia and a combined hemostatic disorder; anemia and thrombocytopenia or thrombopathy resulting in uncontrolled or life-threatening bleeding; and possibly massive transfusion. Stored whole blood is more than 8 hours old: the length of storage depends on the anticoagulant/preservative solution used and varies from 48 hours for 3.8% sodium citrate (no pre-

servative) to 4 weeks for CPD-A1 (citrate, phosphate, dextrose, and adenine) (see Chapter 94). Stored whole blood contains plasma proteins and RBCs but not functional platelets or coagulation factors. A possible indication for SWB is anemia and hypoproteinemia (e.g. chronic gastrointestinal bleeding), although administration of pRBCs and, if there is a clinical need to increase the patient's oncotic pressure, a synthetic colloid or plasma would be appropriate. Given the loss of important blood components with storage, it is much more efficient to separate WB into pRBC and FFP than to store as WB. Also, in anemic patients with underlying cardiac disease, pRBCs would be clearly preferable to WB in an effort to avoid circulatory overload.

Fresh Versus Stored RBCs

Although storage of blood components allows for a readily available supply of RBCs for transfusion, it has been well-documented that during storage RBCs undergo a number of physical and chemical changes, collectively referred to as a “storage lesion,” which reduce RBC function and viability after transfusion.¹⁴ The effects of prolonged storage on RBCs include decreased deformability, which can impede microvascular flow; depletion of 2,3-diphosphoglycerate (2,3-DPG), which shifts the oxyhemoglobin dissociation curve to the left and reduces oxygen delivery; reduction in RBC ATP concentration, which may result in decreased phosphorylation of other proteins or lipid kinases important in maintaining RBC integrity; reduction in RBC-derived nitric oxide bioactivity, which may impair the vasodilatory response to hypoxia; and accumulation of proinflammatory bioactive substances.^{1,14,16,21} Addition of various preservative solutions containing dextrose, adenine, and phosphate (substrates for RBC energy metabolism) improve RBC post-transfusion viability. A study of the effect of storage on canine pRBCs collected in citrate-phosphate-dextrose solution and resuspended in the additive solution Adsol (containing adenine, dextrose, saline, and mannitol) has documented a steady decrease in mean 2,3-DPG concentration from 15.2 μ mol/g Hgb on day 1 to 3.7 μ mol/g Hgb on day 44.²⁵ However, the decrease in 2,3-DPG concentration in stored human and presumably other animal RBCs is a reversible change, with complete restoration of 2,3-DPG levels post-transfusion taking up to 72 hours.²¹ Decrease in RBC 2,3-DPG concentration with storage is not an issue when transfusing cats because feline RBCs contain very low levels of 2,3-DPG and feline Hgb does not require 2,3-DPG for release of oxygen.³

Experimental data in canine models have yielded conflicting results regarding oxygen delivery by fresh compared to stored RBCs. Although a diminished release of oxygen to tissue was demonstrated following administration of stored (21 days) RBCs compared with fresh RBCs in an isolated hind-limb model in the dog,²⁸ a study comparing the effects of administration of autologous stored (21 days) and fresh RBCs in restoring muscular tissue oxygenation after profound isovolemic

hemodilution in dogs did not reveal a difference in tissue oxygenation between the two groups at comparable [Hgb].¹⁹ Currently, there are no clinical data in the dog to document increased morbidity or mortality associated with transfusion of stored rather than fresh RBCs.

There is considerable controversy in human medicine over recommendations for administration of “newer blood” (units stored for 14 days or less) in preference to “older blood” (units stored for more than 14 days). In a single-center, retrospective analysis of outcomes in 6,000 patients who had undergone coronary artery bypass grafting, valve surgery, or both, transfusion of “older blood” (median duration of storage 20 days) was associated with a significantly increased risk of postoperative complications, as well as reduced short-term and long-term survival.¹⁴ On the other hand, a retrospective study evaluating age of transfused RBCs and early outcomes after similar cardiac surgeries in 901 patients found no correlation between duration of storage of RBCs and early adverse outcomes after cardiac surgery.²⁷ A review of nine clinical studies that evaluated the consequences of prolonged RBC storage suggested a possible detrimental clinical effect associated with transfusion of stored RBCs to critically ill patients.²¹ However, one must consider that cardiac surgery and critically ill patients are not representative of all patients in need of RBC transfusions. In light of the consequences of shortening RBC storage time on the blood supply, both sides (for and against use of “older blood”) agree that randomized clinical trials to evaluate the clinical consequences of transfusing older stored RBCs are required before changing blood banking standards for RBC storage time.

ADDITIONAL BENEFITS OF RBC TRANSFUSIONS

Enhanced hemostasis is an often overlooked benefit of RBC transfusions. A correlation between anemia and prolonged bleeding time (BT) has been reported in humans, and correction of the anemia results in shortening of the BT.²³ The effect of Hct on the volume of blood shed during a BT test can be dramatic: a reduction in Hct from 45% to 35% in normal, healthy humans resulted in a greater than two-fold increase in the volume of shed blood.⁶ In a study evaluating the effect of Hct and platelet count on the BT measurement, healthy human volunteers underwent either a 2-unit RBC apheresis procedure followed by return of platelet-rich plasma from both units (resulting in a 15% reduction in the peripheral venous Hct and a 9% reduction in the platelet count) or a plateletpheresis procedure (resulting in a 32% decrease in platelet count and no change in peripheral venous Hct).²² Interestingly, there was no change in BT following the plateletpheresis procedure, yet the RBC apheresis procedure resulted in a 60% increase in the BT, which was subsequently corrected by transfusion of RBCs.²² Furthermore, the acute reduction in Hct following the RBC apheresis procedure was accompanied by a decrease in the shed blood

thromboxane B₂ level at the template BT site, in agreement with results of *in vitro* studies showing that RBCs stimulate thromboxane production by platelets.²² Other potential mechanisms for RBCs leading to an improvement in primary hemostasis include: dispersion of platelets from the center of the blood vessel toward the endothelial cells of the vessel wall, facilitating the platelet-vessel adhesion; shear stress-induced release of ADP, a platelet agonist, from RBCs; and scavenging of endothelial cell nitric oxide by oxidation and by nitric oxide binding to Hgb.²³ In light of the beneficial effects of RBCs on hemostasis, it has been recommended in human medicine that nonsurgical bleeding diatheses in anemic thrombocytopenic patients should be managed with RBC transfusions to increase the Hct to 35% prior to administering platelet transfusions.²³

In addition to providing oxygen-carrying support and improving hemostasis, RBC transfusions are an excellent source of readily bioavailable iron, with 1 mL of pRBCs containing approximately 1 mg of iron. RBC transfusions, therefore, are of benefit in the initial treatment of patients with severe iron-deficiency anemia. However, repeated RBC transfusions to patients with anemia due to causes other than continuous blood loss may place them at risk for developing hemochromatosis, which manifests clinically as organ dysfunction secondary to iron-induced injury. There is a single case report of transfusional hemochromatosis in a dog with pure RBC aplasia that received RBC transfusions every 6–8 weeks for 3 years.¹⁸

ADMINISTRATION

Careful attention to administration is essential to prevent damage of the blood product and harm to the patient. Prior to a RBC transfusion, blood typing and/or crossmatching should be performed to assure RBC compatibility (see Chapter 139). Segments of tubing from the donor blood bag may be used for the blood crossmatch and for quality control and investigation of transfusion reactions (see Chapter 100).

Canine pRBCs stored in a RBC preservative solution such as Adsol can be administered directly, whereas other pRBC products should be diluted by adding 100 mL of saline to the blood bag (or 10 mL of saline to feline pRBCs), thus decreasing the viscosity of the donor blood. Concurrent administration of any drugs or fluids other than physiologic saline through the same catheter should be avoided to prevent blood coagulation and lysis of RBCs induced by contact with calcium-containing solutions and hypotonic solutions, respectively.

Warming of Blood

In the routine administration of RBC products to normovolemic anemic patients, refrigerated blood components do not require warming prior to transfusion; in fact, warming may accelerate the deterioration of stored RBCs and may permit rapid growth of contaminating microorganisms.⁹ However, in patients that are hypo-

thermic or receiving large volumes of blood, refrigerated RBC products should be prewarmed to temperatures between 22°C and 37°C immediately before transfusion to prevent exacerbation or development of hypothermia, the consequences of which may include cardiac arrhythmias and coagulopathies.⁹ Several types of blood warmers are available: thermostatically controlled waterbaths, dry heat devices with electric warming plates, and high-volume countercurrent heat exchange with water jackets.² Such devices are not typically available in veterinary practices. Alternatively, blood units can be warmed by keeping the unit at room temperature for 30 minutes or by running the tubing of the blood administration set through warm water (37°C) during the transfusion. While it is unnecessary (and not recommended) to warm blood for RBC transfusions at conventional rates, in situations requiring blood warming it is necessary to carefully monitor the temperature of the warming system to prevent RBC vesiculation and fragmentation, as well as hemolysis.

Blood Filters and Infusion Devices

Red blood cell transfusions must be administered through a filter designed to remove clots and particles potentially harmful to the patient. Standard blood infusion sets have in-line filters with a pore size ranging from 170µm to 260µm that trap large cells, cellular debris, and coagulated proteins. According to human blood banking standards, a filter may be used to administer 2–4 units of blood to a patient or for a maximum time limit of 4 hours; the combination of a high protein concentration at the filter surface and room temperature conditions promote proliferation of any contaminating microorganisms, and accumulated material slows the rate of flow.² Other blood filters with a pore size of 20–40µm remove microaggregates composed of degenerating platelets, white blood cells (WBCs), and fibrin strands that form in blood after 5 days or more of refrigerated storage. Microaggregate filters are designed primarily for transfusions of RBCs. A pediatric microaggregate blood filter (18µm pore size, priming space <1 mL) (Hemo-Nate Filter, Gesco International, San Antonio, TX) is particularly helpful in administering small volumes of blood (<50 mL WB or <25 mL pRBCs) to cats and small dogs: administering larger volumes of blood could result in hemolysis due to a progressive decrease in pore size as more blood is filtered.

Leukocyte reduction filters have received much attention in human blood banking because of the potential complications in transfusion recipients associated with residual WBCs in cellular blood components, namely febrile nonhemolytic transfusion reactions, human lymphocyte antigen alloimmunization, and cytomegalovirus transmission.² The American Association of Blood Bank Standards defines a leukocyte-reduced RBC product as containing $<5 \times 10^6$ residual donor WBCs.² The frequency of adverse effects attributed to WBCs in blood products has not yet been documented in veterinary patients. It is unlikely that leukoreduction will be cost effective and play an

important role in general RBC transfusions in dogs and cats.

In most instances the blood flow rate through an administration set by gravity alone is adequate to meet a patient's needs. Electromechanical infusion devices that deliver crystalloid or colloid solutions at a controlled rate have been evaluated for administration of blood. Depending on the pump design, some of the infusion devices may induce hemolysis, and some require special plastic disposables or tubing supplied by the manufacturer for use with blood.² In addition to pump design, the degree of hemolysis induced by mechanical pumps depends on the age of the blood (storage time), flow rates, and viscosity of the blood.^{2,20} If considering the use of an infusion pump designed for crystalloid or colloid solutions to administer RBCs, the manufacturer should be consulted first. In the event that a blood flow rate greater than gravity can provide being needed, a specially designed pressure bag that completely encases the blood unit and applies pressure evenly to the bag surface can be utilized: pressures greater than 300 mm Hg may cause the seams of the blood bag to rupture or leak, necessitating close monitoring.² If blood is administered under pressure, large-bore catheters/needles are recommended for venous access to decrease hemolysis.

Route, Volume, and Rate of Transfusion

The intravenous route of administration is recommended for all RBC transfusions; however, intraosseous administration is an excellent alternative if venous access cannot be obtained. Central veins allow for the use of large-bore catheters and thereby more rapid transfusion in the case of hemorrhagic shock. However, peripheral veins may be preferred in animals with an increased bleeding tendency.

The volume of blood to be administered depends on the degree of anemia, the patient's clinical status, and the size of the animal. Similar to the transfusion threshold, a "target PCV," or the desired PCV post-transfusion, has not been clearly defined and is likely to vary depending on the patient's overall condition. It is typically not necessary to transfuse RBCs with the aim of restoring a normal PCV. Dogs and cats with chronic anemia may be cardiovascularly stable with a PCV of 20%, whereas those with an acute onset of anemia and ongoing blood loss or hemolysis may require transfusion to a higher PCV for stabilization. As a general guideline, administration of 2 mL/kg of WB or 1 mL/kg of pRBCs will increase the patient's PCV by 1%, assuming that there is not ongoing hemorrhage or hemolysis. As an alternative to calculating the volume of donor blood required to reach a target PCV, one may administer an average volume of 10 mL/kg of pRBCs or 20 mL/kg of WB to a normovolemic dog and, if deemed necessary based on clinical evaluation of the patient post-transfusion, follow with additional blood. For practical purposes, most anemic cats will initially receive 1 unit of WB (typically 50 mL) or 1 unit of pRBCs (typically 25 mL).

The rate of blood administration depends on the patient's overall condition. In normovolemic anemic patients, the maximum rate of transfusion is 10–20 mL/kg/h to avoid circulatory overload; hypovolemic anemic patients may tolerate replacement of their blood volume as quickly as the blood can be infused. In animals with advanced cardiac disease, the infusion rate should not exceed 2–4 mL/kg/h; pRBCs are preferable to WB in such cases where the transfusion volume tolerated by the patient may be a limiting factor in providing sufficient oxygen-carrying support. In all cases, including those in which the donor and recipient have been blood typed and/or crossmatched, the initial rate of transfusion should be slow while observing for any immediate adverse reactions. In general, a blood transfusion should be complete within 4 hours to ensure administration of functional blood components and to prevent growth of bacteria in the event of contamination.²

Massive Transfusion

Massive transfusion represents a special situation with regard to volume and rate of blood transfusion, as well as blood components administered. Massive transfusion has been defined as transfusion of a volume of whole blood or blood components that is greater than the patient's estimated blood volume within a 24-hour period or replacement of half the patient's estimated blood volume in 3 hours.¹⁰ In a retrospective study of dogs receiving massive transfusion, the mean volumes of pRBCs and FFP administered were 66.5 mL/kg and 22.2 mL/kg, respectively, resulting in a mean plasma:RBC ratio of 1:3.¹⁰ Clinicopathologic changes noted post-transfusion included ionized hypocalcemia and hypomagnesemia and progressive thrombocytopenia and prolongation of prothrombin and activated partial thromboplastin times.¹⁰ There is limited information on massive transfusion in cats.¹⁷ Guidelines for massive transfusion in dogs and cats have not been established. In a retrospective study of massively transfused human trauma patients, 30-day survival was increased in patients with high plasma:RBC ratio ($\geq 1:2$) relative to those with low plasma:RBC ratio ($< 1:2$), resulting in the recommendation that massive transfusion practice guidelines should aim for a 1:1 ratio of plasma:RBCs.⁸

PATIENT MONITORING

Early recognition of transfusion reactions requires careful evaluation of patient's attitude, vital signs, and perfusion (i.e. capillary refill time and pulse quality) before, during, and after a RBC transfusion. Measurement of PCV and total solids pre- and post-transfusion (at a minimum, immediately and at 24 hours) and evaluation of the plasma and urine for the presence of Hgb are recommended. Any unexpected change in attitude, vital signs, or laboratory parameters may be indicative

of an adverse reaction, in which case the RBC transfusion should be stopped immediately, the intravenous line left open with physiologic saline, and other supportive care provided as necessary (see Chapter 100 for a complete discussion of transfusion reactions).

Dogs and cats receiving RBC transfusions frequently have serious underlying diseases, reflected by a high mortality rate, with approximately 60% surviving to hospital discharge.^{4,12,26} However, the benefit of RBC transfusions in providing additional oxygen-carrying support to severely anemic dogs and cats is readily apparent. RBC transfusions can be lifesaving, provided that blood typing and crossmatching are performed to assure RBC compatibility and that donor blood is collected, stored, and administered according to blood banking guidelines.

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Transfusion of Plasma Products

MARJORY B. BROOKS

Plasma Constituents

- Hemostatic Proteins
- Albumin and Immunoglobulins

Plasma Components

- Fresh Frozen Plasma
- Frozen Plasma
- Cryoprecipitate
- Cryosupernatant

Plasma Derivatives and Recombinant Proteins

Indications for Plasma Therapy

- Hemorrhagic Disorders
 - Hereditary bleeding disorders
 - Acquired factor deficiencies

Non-hemorrhagic Disorders

- Hypoproteinemia
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Guidelines for Plasma Administration

- Sources of Plasma Products
- Plasma Product Administration
 - Plasma components
 - Plasma derivatives and recombinant proteins

Adverse Reactions to Plasma Products

- Immune-mediated Reactions
- Non-immune Reactions

Acronyms and Abbreviations

CPD, citrate-phosphate-dextrose; DIC, disseminated intravascular coagulation; FFP, fresh frozen plasma; FP, frozen plasma; IMHA, immune-mediated hemolytic anemia; IMT, immune-mediated thrombocytopenia; IVIG, intravenous immunoglobulin; RBC, red blood cell; rhFVIIa, recombinant human factor VIIa; TRALI, transfusion-related acute lung injury; TRIM, transfusion-related immunomodulation; VWD, von Willebrand disease; VWF, von Willebrand factor.

PLASMA CONSTITUENTS

Plasma contains a myriad of proteins that maintain homeostasis within the body and respond to environmental challenges. The major therapeutic constituents of plasma include hemostatic proteins, albumin, and immunoglobulins. The term “plasma component” is reserved for products prepared by differential centrifugation using blood banking techniques, while “plasma derivative” refers to products prepared by fractionation in manufacturing facilities.^{1,4} The advent of commercial, referral, and academic veterinary blood banks facilitates routine transfusion of animal components in clinical veterinary practice. Although immunoglobulin concentrates are not available at this time, commercial canine and feline albumin derivatives have recently been developed (Animal Blood Resources International, Stockbridge, MI) not available at this time.

Hemostatic Proteins

In addition to all the coagulation factors required for fibrin clot formation, plasma contains platelet adhesive

proteins, potent anticoagulants, and fibrinolytics. The transformation of soluble plasma fibrinogen to insoluble, polymerized fibrin occurs through the action of serine protease coagulation factors (factors II, VII, IX, X, XI, and XII), cofactors (factors V and VIII), and a transglutaminase (factor XIII). Platelet adhesion under conditions of high shear requires the large multimeric glycoprotein, von Willebrand factor (VWF). The major anticoagulant proteins in plasma are antithrombin, protein C, and protein S. Plasma also contains the fibrinolytic enzyme plasminogen and its primary regulatory proteins, antiplasmin and plasminogen activator inhibitor-1. Fibrinogen is the highest concentration hemostatic protein, circulating in plasma at approximately 300 mg/dL.

Albumin and Immunoglobulins

Albumin is the major oncotic protein in plasma and a carrier protein for lipophilic compounds. The immunoglobulin fraction of plasma consists of IgG (up to 85%), IgA, and IgM. The plasma albumin concentration of most species ranges from approximately 2 g/dL to

4g/dL, with immunoglobulins generally present at equivalent or slightly lower concentration. Derivative concentrates, rather than plasma components, are used in human medicine to supply therapeutic levels of albumin and globulins.

PLASMA COMPONENTS

Centrifugation of whole blood sediments the heavier cellular elements from the supernatant plasma. All protein activity and concentration are retained in the plasma if separation occurs within 8 hours of blood collection.¹ This supernatant plasma can be transfused immediately as fresh plasma, but it is usually frozen and stored as fresh frozen plasma (FFP) for subsequent transfusion, or further processed to yield cryoprecipitate and cryosupernatant (Fig. 96.1). The general procedures for preparation of animal components in the United States follow human blood banking guidelines, e.g. collection of whole blood in citrate-phosphate-dextrose (CPD) anticoagulants, initial quick-freeze of separated plasma, and storage temperatures at or below -18 °C.^{1,8}

Fresh Frozen Plasma

Fresh frozen plasma refers to plasma separated from whole blood within 8 hours of collection and quick-frozen within 1 hour of separation. It contains essentially all the hemostatic proteins, albumin, and globulin

as the plasma from which it was prepared. The term FP24 is used in human blood-banking for plasma separated and frozen within 24 hours from collection. Compared to FFP, this product has diminished activity of labile factors, but is approved as a source of coagulation factors for patients with moderate, combined factor deficiencies.^{2,12} Veterinary studies have confirmed the retention of functional hemostatic proteins in FFP units for up to 1 year of storage.^{15,17}

Frozen Plasma

Frozen plasma (FP) refers to FFP units stored longer than 1 year or thawed FFP units stored under refrigeration for more than 24 hours before transfusion. Hemostatic proteins retain variable activity in FP, and the product does not supply replacement levels of the most labile coagulation factors, factors V and VIII. FP is a source of albumin and globulins.

Cryoprecipitate

Cryoprecipitate is prepared by slowly thawing FFP at temperatures between 1 to 6 °C, followed by centrifugation to sediment the large, cold-insoluble proteins.^{1,12} The resultant cryoprecipitate fraction is then refrozen for storage within 1 hour of separation. “Units” of cryoprecipitate may be defined based on the volume of starting FFP, or fibrinogen or factor content. Cryoprecipitate contains fibrinogen, the VWF-factor

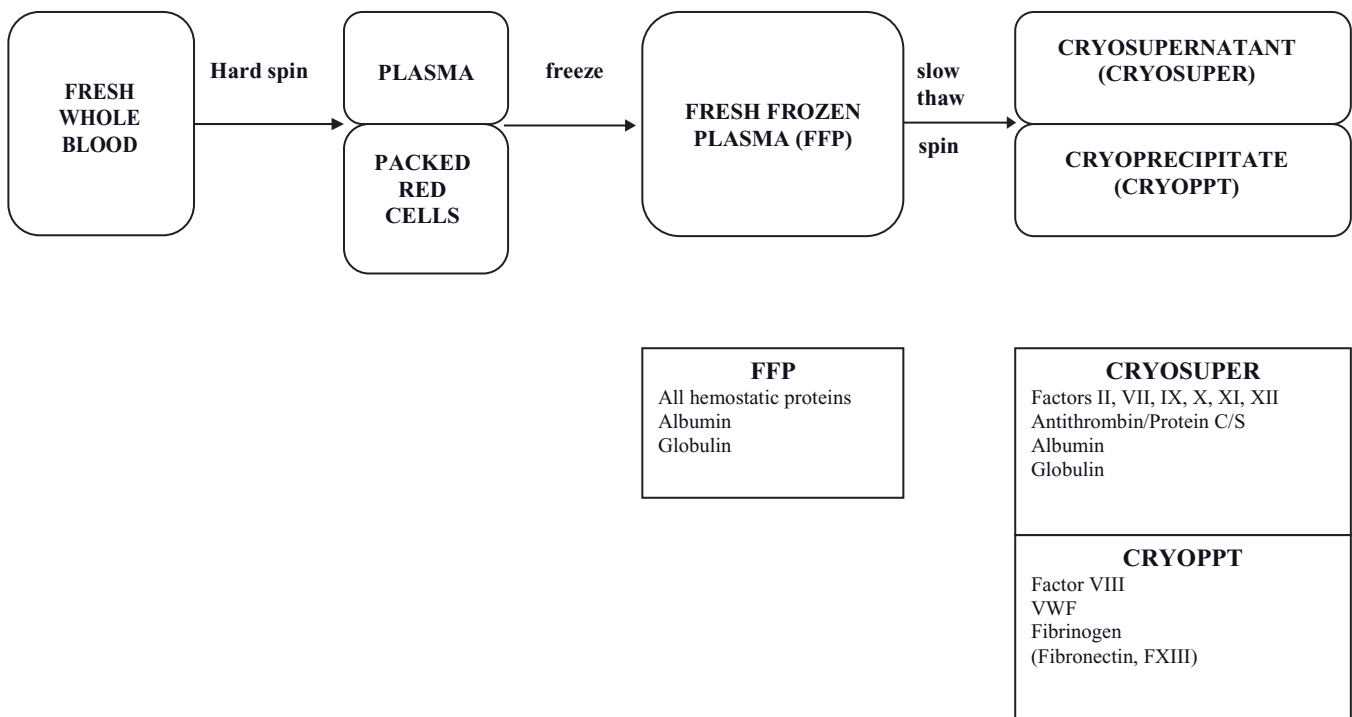


FIGURE 96.1 Flow chart of plasma component processing and composition.

VIII complex, factor XIII, and fibronectin. The process of cryoprecipitation yields approximately 50% factor activity, in one-tenth volume, of the starting FFP. Volume reduction is the major advantage of cryoprecipitate transfusion; therapeutic levels of factors are attained within minutes over the course of a single bolus infusion. Cryoprecipitates are stable for up to 1 year of storage and are often used for animals as sources of VWF and factor VIII.

Cryosupernatant

Cryosupernatant is the plasma component remaining from production of cryoprecipitate.^{1,12} This product is deficient in fibrinogen, VWF-factor VIII, and other cold-insoluble proteins, but retains proteins such as albumin and most hemostatic proteins and immunoglobulins. Cryosupernatants are often used as a source of the vitamin K-dependent coagulation factors (factors II, VII, IX, and X) and are stable for this purpose for at least 1 year of storage.

PLASMA DERIVATIVES AND RECOMBINANT PROTEINS

Human apheresis plasma provides most of the source material for commercial manufacture of pharmaceutical protein derivatives.⁶ Human albumin, immunoglobulin, and factor concentrates (factor VIII and VWF) are among the most commonly used derivatives in medical practice. The first production methods developed in the 1940s and 1950s (Cohn's fractionation) separated constituent proteins based on differential solubility under conditions of varying temperature, pH, and ethanol concentration. Subsequent advances include the use of chromatography and immunopurification processing techniques. The final protein concentrates are supplied as solutions or lyophilized powders with long (2–3 year) shelf-lives at room temperature. To ensure product safety, the manufacturing process of human protein concentrates incorporates donor and plasma screening and highly effective pathogen inactivation procedures.

In addition to high purity factor VIII and IX concentrates, recombinant human factor VIII and IX products are approved to prevent and control hemorrhage in hemophilic patients. Although recombinant factors carry no risk of blood-borne disease transmission, they do not prevent development of inhibitory alloantibodies, a severe complication of replacement therapy. Recombinant human factor VIIa (rhFVIIa) was developed as a bypassing agent to treat hemophiliacs and other patients with acquired coagulation inhibitors.⁹ At supra-physiologic levels, rhFVIIa generates thrombin independent of factors VIII and IX by directly activating factor X on the surface of procoagulant platelets.

INDICATIONS FOR PLASMA THERAPY

Plasma components are the mainstay for treatment of hemorrhagic disorders caused by hemostatic protein deficiencies. Plasma components are also transfused to animals as a source of albumin and immunoglobulins because of the lack of allogeneic derivative protein concentrates.¹⁰ The therapeutic goal for each transfusion should be clearly established in order to select the most specific replacement product (Table 96.1). This approach maximizes the use of scarce plasma resources and helps minimize recipient exposure to foreign antigens.

Hemorrhagic Disorders

Hereditary Bleeding Disorders

Plasma components are indicated to control active hemorrhage and for preoperative prophylaxis for animals with hereditary coagulation factor deficiencies and von Willebrand disease (VWD) (see Chapters 81, 86, and 90). In many cases, appropriate component therapy will eliminate the need for red blood cell (RBC) transfusion with its inherent risks of sensitization and adverse reaction to RBC antigens.

Von Willebrand disease, among the most common hereditary bleeding disorders of dogs, horses, and people, is often managed with plasma component therapy. The most clinically severe forms of VWD (types

TABLE 96.1 Plasma Components and Plasma Derivatives

Product	Dosage Guidelines	Indications
Fresh Frozen Plasma	6–12 mL/kg q8–12 h	Inherited and acquired coagulation factor deficiencies, von Willebrand disease, hypoproteinemia, hypoglobulinemia
Cryoprecipitate ^a	1 unit/10 kg q4–12 h	Hemophilia A, von Willebrand disease, hypofibrinogenemia
Cryosupernatant	6–12 mL/kg q8–12 h	Hemophilia B, hereditary deficiencies of Factors II, VII, X, XI, vitamin K deficiency, hypoproteinemia, hypoglobulinemia
Frozen plasma	12–20 mL/kg q12–24 h	Hypoproteinemia, hypoglobulinemia
Human albumin solution ^b	0.5–1.0 g/kg	Hypoproteinemia
Human intravenous immunoglobulin ^b	0.5–1.0 g/kg	Refractory immune-mediated disorders
Recombinant human FVIIa ^b	35–120 µg/kg	Factor bypassing agent, surgical hemostasis

^a1 unit cryoprecipitate prepared from 200 mL fresh frozen plasma.

^bAdministration of human protein products may induce severe adverse reactions in animals.

2 and 3 VWD) are characterized by dysfunctional or absent VWF protein, respectively. Type 1 VWD is clinically variable, associated with a quantitative VWF deficiency. Patients with severe forms of VWD invariably require replacement therapy if they undergo invasive procedures and are subject to spontaneous mucosal bleeds that may persist without transfusion. Transfusion requirements are generally lower for type 1 VWD; however, each patient's risk of hemorrhage should be assessed based on severity of VWF deficiency, prior history of abnormal bleeding, presence of concurrent disorders, and the nature and site of surgery or trauma. The highest potency VWF plasma component is cryoprecipitate; however, FFP can be transfused as a source of VWF if cryoprecipitate is unavailable.

Hemophilia occurs in many different breeds and species. There are two clinically indistinguishable forms: hemophilia A (factor VIII deficiency) is much more common than hemophilia B (factor IX deficiency). Both are X-linked recessive traits, and, therefore, signs of a bleeding disorder are seen predominantly in males. Heritable coagulopathies caused by deficiencies of fibrinogen and factors II, VII, IX, X, and XI have also been identified in animals. These autosomal traits are generally restricted to a single breed or line; however, *de novo* and low frequency mutations can be propagated widely if asymptomatic carriers are bred. Transfusion requirements vary among hereditary factor deficiencies. Clinical expression of a bleeding tendency depends on the physiologic role of the deficient factor and the severity of factor deficiency. For example, hemophilia A and B often manifest as clinically severe coagulopathies, whereas most patients with factor VII deficiency have no signs of a bleeding tendency (see Chapter 86). Hemophilia A and fibrinogen deficiency or dysfunction (dysfibrinogenemia) respond to cryoprecipitate or FFP, if cryoprecipitate is unavailable. All other hereditary factor deficiencies can be treated with FFP or cryosupernatant.

Acquired Factor Deficiencies

Acquired coagulopathies are caused by simultaneous deficiencies of many factors rather than specific deficiency of a single factor. Acquired coagulopathies are more common than hereditary disorders and often develop in patients with vitamin K deficiency, liver disease, disseminated intravascular coagulation (DIC), and massive blood loss treated with large-volume fluid replacement (see Chapter 85).

Transfusion is required for vitamin K-dependent coagulopathies if fulminant hemorrhage develops in critical sites, e.g. respiratory tract or central nervous system, since vitamin K replacement therapy corrects coagulopathy only after a period of 12–24 hours. Cryosupernatant is the most cost-effective plasma component to supply the vitamin K-dependent factors (factors II, VII, IX, X), but FFP is an acceptable alternative (Table 96.1).

In addition to coagulation factors and fibrinogen, the liver is the sole site of synthesis of plasminogen, anti-

coagulant proteins, protease inhibitors, and albumin. While FFP contains all these proteins, volume overload can limit the efficacy of FFP replacement therapy.^{2,10,12} FFP transfusion is useful, however, for short-term replacement of hemostatic levels of coagulation factors in patients with liver failure. FFP transfusion, given at a ratio of 1:1 with packed RBCs is also recommended to manage dilutional coagulopathy in humans with massive blood loss. Controlled studies of DIC in humans have not shown consistent benefit of FFP or factor concentrates in improving clinical outcomes.^{2,3} Therefore, use of FFP is generally restricted to DIC patients with signs of hemorrhage caused by severe fibrinogen and factor depletion.

Non-hemorrhagic Disorders

Hypoproteinemia

Hypoproteinemia develops in numerous disease conditions including vasculitis, protein losing disorders, hepatic failure, and peritonitis. In medical practice, albumin concentrates are used to restore the oncotic properties and carrier functions of albumin.⁵ Antigenic differences among species, however, render human albumin immunogenic, resulting in life-threatening reactions in healthy dogs receiving pharmaceutical human albumin solutions.^{7,14} In light of these findings, off-label use of human albumin solutions for veterinary patients should be carefully considered and proceed only after obtaining owners' informed consent. The plasma components FFP and cryosupernatant contain albumin and are potential sources for veterinary replacement therapy. However, high volume transfusions (20–25 mL/kg) of these components are predicted to obtain calculated increments of 0.5g/dL plasma albumin in recipients. Clinical trials are needed to determine whether plasma component transfusions can improve clinical outcomes in hypoproteinemic veterinary patients.

Immune Disorders

Human intravenous immunoglobulin (IVIG) concentrates were initially developed for treating immunodeficiencies; however, these products are now used to modulate the immune response for diverse human disease syndromes including immune cytopenias, myasthenia, rheumatic disease, and immune-mediated dermatopathies.⁴ The clinical use of human IVIG in animals includes management of refractory immune-mediated disorders, including immune-mediated hemolytic anemia (IMHA), immune-mediated thrombocytopenia (IMT), pemphigus foliaceus, and cutaneous drug eruptions.¹³ The immunoglobulin content of plasma components does not supply supraphysiologic levels of immunoglobulins for immunosuppression; however, FFP (and cryosupernatants) can serve as sources of immunoglobulin replacement therapy for neonates with failure of passive transfer, and to augment response to infectious agents.

TABLE 96.2 Sources of Animal Plasma Components^a

Supplier	Species	Website
Animal Blood Resources International	Dogs, cats, llama, cow, small ruminants	http://www.abrint.net/
Eastern Veterinary Blood Bank	Dogs	http://www.evbb.com/
Hemopet	Dogs	http://www.hemopet.org/
Hemosolutions	Dogs	http://www.hemosolutions.com/
Lonestar Veterinary Blood Banks	Dogs	http://www.lonestarbloodbank.com/
Northwest Veterinary Blood Bank	Dogs, cats	http://www.northwestbloodbank.com/
Plasvacc	Dogs, horses	http://www.plasvacc.com/
Rocky Mountain Blood Services	Dogs, cats	http://rockymountainbloodservices.com/
The Pet Blood Bank	Dogs	http://www.petshelpingpets.com/
The Veterinarians Blood Bank	Dogs	http://www.vetbloodbank.com/

^aInformation current 2009.

GUIDELINES FOR PLASMA ADMINISTRATION

Sources of Plasma Products

The number of commercial veterinary blood banks has more than doubled over the past 5 years to meet rising demands for animal blood products (Table 96.2), in addition to the advent of local and university blood banks. The species and selection of plasma components supplied by different commercial blood banks varies.

Guidelines for screening canine and feline donors have been published recently;¹⁶ however, nationwide standards do not exist for unit definition, potency, or processing techniques to prepare plasma components. Information on quality assurance, quality control, and specific dosage and handling recommendations must be obtained from each supplier.

Plasma Product Administration

Plasma Components

Plasma components are prepared in sterile plastic bags and then stored and shipped frozen in individual boxes.^{1,11} Plasma bags are brittle and must be handled carefully to prevent breakage. All products should be stored at -20°C or lower and then warmed to 37°C in a water bath or incubator just before transfusion. Unopened bags can be refrozen without significant loss of factor activity when kept at 37°C for up to 1 hour, but any product remaining in opened bags should be discarded if not transfused within 3–4 hours.

Intravenous transfusion of plasma components is the preferred route of administration; however, intraosseous transfusion may be indicated in emergency situations when attempts at vascular access have failed. Catheter sites for transfusion should be aseptically prepared and the catheters reserved for plasma component administration (or flushed with sterile saline before and after plasma transfusion). Plasma components should be infused through blood filters and should not be mixed with other fluids, drugs, or additives to prevent contamination, precipitation, or clot formation.

In the author's experience, routine pretreatment with corticosteroids or antihistamines is not required before

plasma component transfusion. Plasma products should be infused at a slow initial rate of 1–2 mL/minute and the recipient closely monitored for signs of transfusion reaction. Maintenance of this rate is advisable for patients at risk for volume overload (e.g. puppies, kittens, cardiac patients). Transfusion rates of 3–6 mL/minute (to total transfusion time of 1.5–2 hours) are generally well tolerated for more rapid factor replacement. Low volume cryoprecipitate can be transfused as a slow intravenous bolus over a period of 10–20 minutes.

The dose and frequency of transfusion vary for different products and different disease conditions (Table 96.1). In general, FFP, FP and cryosupernatant are transfused at 10–12 mL/kg, with an interval of 6–8 hours between doses to prevent volume overload. Cryoprecipitates are supplied in arbitrary "units" defined by each blood bank and should be transfused, therefore, according to the suppliers' recommended dosages. The best strategy for rapidly restoring hemostasis in patients with severe factor deficiencies is to initiate high dose and short interval component transfusion. The high end of dosage range is also appropriate for preoperative prophylaxis. The dose can be decreased and transfusion interval increased after hemostasis is restored. While coagulation assays and point-of-care monitors aid in assessing patient response (Fig. 96.2), clinical assessment and confirmation of rising or stable hematocrit are important parameters to guide subsequent transfusion.³ Patients with severe factor deficiencies usually require more than a single preoperative transfusion to undergo surgery or to control hemorrhage severe enough to cause blood loss anemia. A second (postoperative) transfusion should be given routinely, with access to additional components as needed. Transfusion over a maximum period of 3 days is usually sufficient to support hemostasis and postoperative wound healing in patients with even the most severe factor deficiencies.

Plasma Derivatives and Recombinant Proteins

Administration of human albumin, immunoglobulin, and rhFVIIa has been reported in dogs; however, treatment trials adequately powered to judge clinical effi-

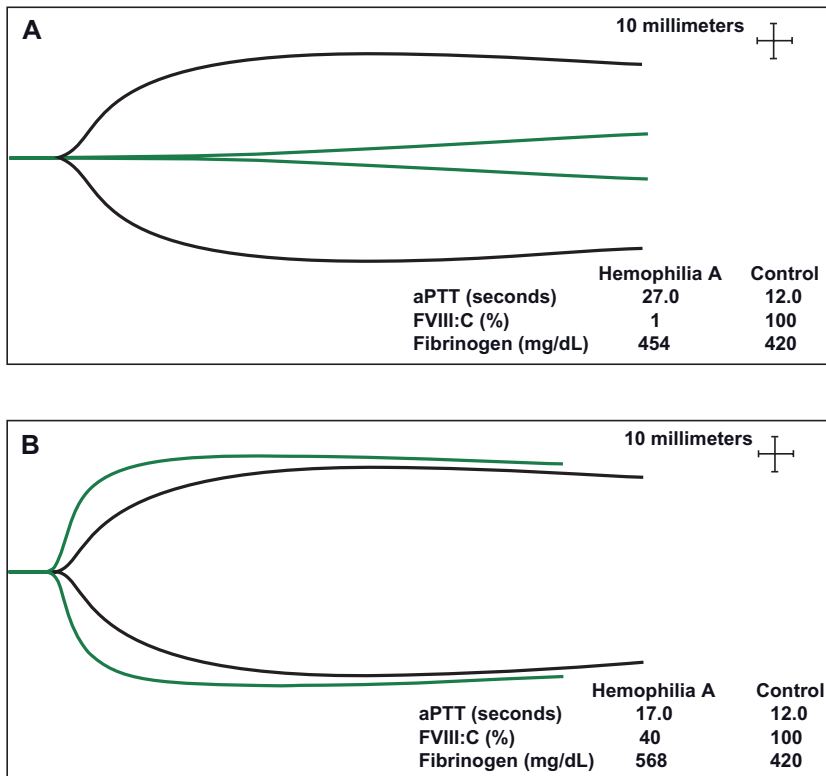


FIGURE 96.2 Response to cryoprecipitate in a dog with severe hemophilia A. Cryoprecipitate was transfused to a hemophiliac dog preoperatively and postoperatively for cutaneous mass excision. Citrate-anticoagulated blood samples were collected before transfusion and 45 minutes after postoperative transfusion to monitor activated partial thromboplastin time (aPTT), factor VIII coagulant activity (FVIII:C), fibrinogen, and thromboelastography (TEG). TEG was performed without activating agents. In each panel, the hemophiliac dog's TEG tracing (green line) is superimposed over a normal dog tracing (black line). Inset tables display aPTT, FVIII:C, and fibrinogen values. **(A)** Pre-treatment TEG depicts minimal fibrin formation in the absence of factor VIII. **(B)** Post-treatment TEG depicts a marked increase in the rate and tensile strength of the fibrin clot formed in the presence of hemostatic levels of factor VIII.

cacy have not yet been performed. In a recent case review,¹⁴ a pharmaceutical 25% human albumin solution was infused (after dilution in sterile saline to a concentration of 10% albumin) to critically ill dogs over a 12 hour interval to deliver a total albumin dosage of 0.5–1.25 g/kg. This dosage regimen produced a median increase in serum albumin of 1.1 g/dL; however, approximately one-quarter of the recipients developed complications, including 4% (3 of 73) with severe delayed immune reactions. Human IVIG has been used in the dosage range of approximately 0.5–1.5 g/kg for refractory IMHA and IMT in dogs.¹³ Cytopenias generally resolved in more than two-thirds of the dogs treated with a single IVIG dose. The hemostatic effects of rhFVIIa have been demonstrated in research dogs with hereditary factor deficiencies (hemophilia A, hemophilia B and factor VII deficiency), at dosages ranging from 5 to 30 µg/kg.⁹ Pending additional veterinary studies, any potential benefit of human plasma-derived or recombinant concentrates must be weighed against their high cost and cross-species immunogenicity.

ADVERSE REACTIONS TO PLASMA PRODUCTS

All plasma recipients should be observed during transfusion, with minimum monitoring of temperature, pulse, and respiration before and after transfusion. Based on the indication for plasma therapy, the appropriate samples to monitor coagulation status, hemat-

ocrit, or protein concentration should be collected before and after transfusion to gauge recipient response. Plasma product administration should be stopped immediately and the product saved for analysis if the recipient displays any signs of discomfort or change in clinical status (see Chapter 100).

Immune-mediated Reactions

Plasma components and concentrates are acellular but contain potentially immunogenic proteins and other compounds (e.g. anticoagulants) capable of eliciting immune reactions. Acute allergic reactions characterized by urticaria, erythema, and facial edema are among the most common reactions and may occur during a first transfusion, or in patients previously transfused many times. Signs typically manifest within the first 30–60 minutes of infusion. The antigens or factors responsible for these reactions have not been defined, but most patients respond quickly if the transfusion is discontinued and they are given antihistamines and/or short-acting steroids. Cats have clinically significant alloantibodies to foreign RBC antigens. Therefore, cats should be transfused only with type-matched plasma products. A minor cross-match (recipient RBCs plus donor plasma component) can be performed in any species to detect hemolysins and agglutinins before transfusion. The off-label use of pharmaceutical agents containing human proteins or any cross-species plasma transfusion carries an inherent risk of potentially fatal acute and/or delayed immune reactions.

The acronyms TRALI (transfusion-related acute lung injury) and TRIM (transfusion-related immunomodulation) refer to deleterious antibody and/or cytokine-mediated plasma component reactions.⁸ TRALI is attributed to alloantibodies in donor plasma activating recipient granulocytes which results in severe pulmonary edema. TRIM is an immunosuppressive syndrome caused by donor leukocytes or leukocyte-derived intermediaries inducing an increased susceptibility to cancer recurrence or bacterial infection in recipients.

Non-Immune Reactions

Non-immune reactions to plasma products are caused by improper collection, storage, or administration procedures or by disease transmission from infected donors.^{2,11} Volume overload is a common non-immune reaction caused by too rapid rate or too high volume transfusion. Clinical signs of this reaction include emesis, vocalization, and dyspnea. Signs of respiratory distress may also develop secondary to transfusion of unfiltered plasma products containing fibrin clot fragments or precipitates formed by plasma contact with calcium or glucose-containing solutions. Although RBC and platelet products are more frequently implicated in disease transmission, appropriate donor screening and careful attention to aseptic procedures for catheter placement are important to minimize risk of infection. Blood banking recommendations to minimize plasma component contamination include avoidance of transfusion times longer than 4 hours and prohibiting introduction of any drugs or solutions into the plasma product.

The demand for animal plasma components and derivatives is likely to intensify as veterinarians provide increasingly complex medical and surgical services. The challenge remains for clinicians and researchers to develop mechanisms for evaluating the risks and ben-

efits of plasma transfusion and protocols for optimizing plasma usage and clinical outcomes.

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Platelet and Granulocyte Transfusion

ANTHONY C.G. ABRAMS-OGG

Platelet Transfusion

Assessment of Platelets for Transfusion

Whole Blood-Derived and Apheresis Platelets

Storage of Platelet Products

Chilled Platelets

Cryopreserved Platelets

Lyophilized Platelets and Platelet Substitutes

Transfusion of Platelet Product

Fresh Whole Blood

Platelet-rich Plasma and Platelet Concentrate

Platelet-poor Blood Components

Platelet Transfusion Reactions

Granulocyte Transfusion

Acronyms and Abbreviations

AABB, American Association of Blood Banks; CoE, Council of Europe; DMSO, dimethyl sulfoxide; FWB, fresh whole blood; G-CSF, granulocyte colony-stimulating factor; GC, granulocyte concentrate; IMT, immune-mediated thrombocytopenia; PC, platelet concentrate; PPR, percent platelet recovery; PRP, platelet-rich plasma; RBC, red blood cell; RT, room temperature; VWF, von Willebrand factor.

PLATELET TRANSFUSION

Platelet transfusion is indicated for the control of bleeding due to severe thrombocytopenia or thrombopathia. Platelet transfusion has not been as widely used in veterinary medicine as red blood cell (RBC) and plasma transfusion, and most reports concern platelet transfusion associated with experimental hematopoietic stem cell transplantation in dogs. However, with the use of more aggressive anticancer therapy and the increased willingness to treat complex hematologic disorders, there is an increasing need for platelet-rich blood products. These products include fresh whole blood (FWB), platelet-rich plasma (PRP) and platelet concentrate (PC). The latter may be prepared by centrifugation of FWB or by plateletpheresis, the selective removal of platelets from the donor's blood via an automated cell separator with return of RBCs and plasma to the donor.

ASSESSMENT OF PLATELETS FOR TRANSFUSION

Methods for the collection and storage of human platelets are being continuously evaluated; results are compared on the basis of in vitro and in vivo

tests of platelet quantity and quality in PRP and PC.

Platelet *quantity* is typically determined using standard methods of platelet counting for hemograms, but automated hematology analyzers are not specifically calibrated for this purpose and results will vary with the method used (see Chapter 135). Platelet *yield* from centrifugation is calculated by dividing the number of platelets in the final product by the number of platelets in the initial product. Platelet *survival during storage* is calculated by dividing the number of platelets remaining at the end of the storage period by the number present at the beginning. American Association of Blood Banks (AABB) standards specify that human PC production by centrifugation should result in $\geq 5.5 \times 10^{10}$ platelets in $\geq 90\%$ of units,¹⁰ while Council of Europe (CoE) standards specify $\geq 6.0 \times 10^{10}$ platelets in $\geq 75\%$ of units.¹⁵ Plateletpheresis should result in $\geq 3.0 \times 10^{11}$ (AABB) or $\geq 2.0 \times 10^{11}$ (CoE) platelets in $\geq 90\%$ of units.^{10,15}

During platelet collection and storage, deleterious physical and metabolic changes referred to as the *storage lesion* occur, which are greater than what is expected with senescence alone. They correlate to some extent with decline in pH, and, while there is not a direct correlation between pH and platelet health, pH remains an important parameter. The AABB standards specify a

pH > 6.2 in 90% of units at the end of the allowable storage period; CoE standards specify a pH of 6.4–7.4 in all units.^{10,15}

Many of the tests discussed in Chapter 142 to evaluate platelet function *in vitro* have been used in a largely unsuccessful effort to predict post-transfusion behavior.³² The poor predictive value occurs at least in part because the metabolic stress in stored platelets may be reversible after transfusion.⁴⁰ The most useful tests are response to hypotonic stress and evaluation of platelet morphology, including “shape change” in response to agonists, morphology scoring using light or electron microscopy, and evaluation of swirling in the blood bag with the unaided eye. Of these, only the latter is practical as a routine test in the clinic; the others are used primarily in a research capacity. Given the paucity of evidence-based data on clinical platelet transfusion in dogs and cats, the following tests are recommended for routine use: (1) measurement of platelet count in the product prior to transfusion; (2) assessment of swirling in PRP and PC; and (3) measurement of pH in PRP and PC after storage.

Platelet *in vivo* viability refers to platelet survival following transfusion. This is determined by radiolabeling or biotinylating platelets and measuring a decrease in labeled platelets over time (see Chapter 134).^{28,47} In the clinical setting, platelet viability is determined by measuring the incremental increase in platelet counts following transfusion. One-hour and 24-hour platelet increments are standard measurements; 10 minute increments have also been used.³⁶

The final proof of platelet quality is control of hemorrhage in experimental or naturally-occurring thrombocytopenia or thrombopathia.²⁷ Improved hemostasis may be documented by subjective observation of the bleeding patient and comparison of pre- and post-transfusion bleeding time, Platelet Function Analyzer-100 (Siemens Healthcare Diagnostics, Deerfield, IL) closure time, and whole blood (WB) clotting assays using thromboelastography, resonance thrombography, or a Sonoclot analyzer (Sienco, Arvada, CO) (see Chapters 138 and 142). In addition to the quality of the PRP or PC, improved hemostasis is dependent on the number of transfused platelets and recipient factors affecting platelet function and viability.

WHOLE BLOOD-DERIVED AND APHERESIS PLATELETS

Preparation of PRP and PC from FWB is described in Chapter 94. Platelet-rich plasma and PC should be labeled with donor blood type as these products invariably have some RBC contamination. As indicated above, the main benefit of apheresis PC over whole blood-derived PC or PRP is a greater total platelet yield ($\geq 3.0 \times 10^{11}$ vs. $\geq 5.5 \times 10^{10}$ platelets per unit).

Plateletpheresis has been performed successfully in dogs (10–28 kg body weight) using both automated continuous^{4,11} (Fresenius AS 104, Fresenius AG, Bad Homburg, Germany; and COBE Spectra, Caridian BCT,

Lakewood, CO) and discontinuous^{24,49} (Haemonetics MCS Plus and Haemonetics 30, Haemonetics Corporation, Braintree, MA) flow centrifugation. Blood is typically collected through a 14–16 gauge jugular venous catheter, and platelet-poor blood is returned via a peripheral venous catheter. Platelet concentrate volume and platelet yield from plateletpheresis has ranged from 120 to 322 mL and 1.26×10^{11} to 4.5×10^{11} platelets/PC, respectively.^{4,11,24,49} Collection time is approximately 1–2 hours, depending on desired platelet yield.^{4,11} *In vivo* platelet recovery, defined as the percentage of transfused platelets (⁵¹Cr-radiolabeled or biotinylated) that survived in the recipient, has been described as approximately 80% at 1–2 hours post-transfusion.^{6,49} The transfused apheresis platelets have a mean half-life of 3.5–3.8 days and a lifespan of 7–9 days.^{6,49}

The main adverse effects of plateletpheresis on the canine donor are thrombocytopenia and hypocalcemia. Thrombocytopenia is usually mild, with most counts remaining above 100,000/ μ L and returning to baseline by 4–6 days. Ionized calcium levels drop during plateletpheresis because of citrate toxicosis during reinfusion of PPP and RBCs to the donor. Clinical signs of hypocalcemia were only noted in 3 of 14 dogs in one study and were generally mild, but occurred despite intravenous calcium supplementation.¹¹ There were no long-term side effects noted in any study.

STORAGE OF PLATELET PRODUCTS

Veterinary hospitals with blood component-producing capabilities usually prepare PRP and PC on an as-needed basis, but PRP and PC may be stored for 5–7 days at room temperature (RT, 20–24 °C) with continuous or intermittent agitation; FWB may be stored for up to 8 hours at RT (see Chapter 94). Due to the increased risk for bacterial proliferation at RT storage, there has been much interest in evaluating chilled (4 °C) storage of platelets. In addition, cryopreservation and lyophilization of platelets have been evaluated as long-term storage options for platelets. Commercial availability of cryopreserved and lyophilized canine PCs (Animal Blood Resources International, Stockbridge, MI) provides an immediate source of platelets for transfusion in clinical practice.

Chilled Platelets

Prior to 1969 human platelets were refrigerated at 4 °C, similarly to RBCs.³⁵ However, evidence of reduced *in vivo* viability after 8–24 hours of chilling prompted the current standard of preparation and storage at RT.^{10,20,35,44} During the first 24 hours of refrigeration platelets actually had normal-to-increased *in vitro* aggregation responses and demonstrated immediate *in vivo* hemostatic efficacy in humans with thrombocytopenia and aspirin-induced thrombocytopenia.^{8,20,48} However, after 24 hours of refrigeration *in vitro* aggregation, *in vivo* viability, and hemostatic efficacy rapidly dimin-

ished.^{8,20,44} The mechanism of reduced *in vivo* viability has been recently elucidated: chilling platelets clusters their von Willebrand factor (VWF) receptors, eliciting recognition of platelets by hepatic macrophage complement type 3 receptors, with subsequent phagocytosis.³⁰ The clustering does not impair functional binding of VWF.³⁰ Galactosylation of 2-hour chilled murine platelets blocked complement type 3 receptor-mediated phagocytosis and restored platelet *in vivo* viability, but unfortunately did not prevent rapid clearance of either murine or human platelets chilled for 48 hours.⁵¹ There are no published reports on *in vivo* viability of chilled canine platelets. However, in light of the effect of chilling on survival of both murine and human platelets, refrigerated storage of canine platelet-rich products (FWB, PC, PRP) cannot be recommended at this time without studies documenting adequate post-transfusion survival.

Cryopreserved Platelets

Another approach to prolong shelf-life and minimize bacterial growth is cryopreservation, which is approved for human PC and has been used to store canine PC for up to 2 years. Platelets are cryopreserved using a cryoprotectant solution in approved plastic bags at a freezing rate of 1–3°C per min. In general, cryopreserved human platelets have reduced function and viability compared to platelets stored at RT.¹⁰ In unrelated studies, canine platelets cryopreserved in 6% dimethyl sulfoxide (DMSO) have been shown to have impaired *in vitro* function based on marked reduction of aggregation, although cryopreserved platelets can be activated *in vitro* as demonstrated by thrombin-stimulated P-selectin expression.^{5,6,22} Furthermore, cryopreserved canine platelets have reduced *in vivo* recovery (~50%) and survival (half-life ~ 2 days) compared to fresh canine platelets,^{6,49} but were reported to have hemostatic efficacy in lethally irradiated dogs.⁴⁹ These results may reflect the poor predictive value of *in vitro* tests and/or the clinical benefit of transient platelet support. Canine platelets cryopreserved in polyethylene glycol-DMSO were also anecdotally effective in controlling bleeding in Basset hounds and Otterhounds with thrombopathia (W.J. Dodds, personal communication).³⁹ An augmented cryoprotectant containing 2% DMSO and Thrombosol (a mixture of amiloride, adenosine, and sodium nitroprusside, second-messenger effectors that inhibit platelet activation) improved human, but not canine, platelet viability in comparison to 6% DMSO.⁶

Lyophilized Platelets and Platelet Substitutes

Lyophilization has also been reported for long-term storage of canine platelets and for preparing infusible platelet membranes, a platelet transfusion substitute, using a proprietary freeze-thaw process.^{9,13} Other potential platelet transfusion substitutes include liposome-encapsulated platelet membrane fractions

and polymerized albumin particles carrying fibrinogen gamma-chain dodecapeptide.^{37,42}

TRANSFUSION OF PLATELET PRODUCT

Platelet transfusion is most useful in the management of bleeding in patients with severe thrombocytopenia due to decreased platelet production, where transfused platelet lifespan is normal. It is clinically most rewarding when prompt marrow recovery is anticipated, e.g. following anticancer chemotherapy. Platelet transfusion is less beneficial in disseminated intravascular coagulation (increased platelet consumption), but may be given if the patient is bleeding. Platelet transfusion is least beneficial in immune-mediated thrombocytopenia (IMT) because transfused platelets may be rapidly destroyed; however, platelet transfusion has been useful in some cases and should be considered when there is critical hemorrhage.^{1,12} Platelet transfusion is beneficial in most cases of bleeding due to thrombopathia.

Platelet transfusion triggers and doses have been reviewed.¹ A platelet transfusion may be *therapeutic*, given with intent to stop bleeding, or *prophylactic*, given with intent to prevent bleeding. Although controversial, prophylactic transfusion is the most common practice in human medicine, with transfusion triggers of 10,000/ μ L in the absence of other risk factors for bleeding, 20,000/ μ L with other risk factors, and 50,000–100,000/ μ L prior to invasive procedures. Prophylactic transfusions increase the use of platelet products. Most transfusions given to dogs and cats are therapeutic. The latter approach requires close patient monitoring and a readily available platelet product.

Platelet dose is also controversial.¹ A strategy of higher-dose, less frequent transfusions may be used, versus one of lower-dose, more frequent transfusions. Platelet survival is increased at higher platelet counts, supporting the first strategy, which is particularly applicable if transfusions are given on an outpatient basis. However, this may increase the total number of units used. Economics and resources often dictate a minimal transfusion dose in animals (especially large dogs), which are usually hospitalized and transfused therapeutically, promoting the latter strategy. It is often not necessary to raise the platelet count by large increments – critical spontaneous hemorrhage can usually be prevented by transfusions to keep the platelet count above 10,000–15,000/ μ L.

As with RBC and plasma components (see Chapters 95 and 96), platelet transfusions should be given using standard blood administration sets with 170 μ m filters. Transfusion sets should be free of latex, which can bind platelets. Most infusion pumps may be used without damaging platelets, but the manufacturer should be consulted.

The success of a platelet transfusion is judged by control of hemorrhage and by comparing the expected and measured 1-hour platelet increment. If the transfusion was beneficial, active hemorrhage will be reduced.

If bleeding is well-controlled, existing petechiae and ecchymoses will fade over 12 hours. The expected 1-hour post-transfusion platelet count may be calculated as follows: expected 1-hour platelet count = platelet count pre-transfusion + expected 1-hour platelet increment, where expected 1-hour platelet increment = unit platelet count \times unit volume \times percent platelet recovery (PPR)/estimated blood volume. The PPR corrects for a fixed loss of transfused platelets due to splenic sequestration and other factors.^{17,46} If the measured platelet count is much below the expected count, there is accelerated destruction, consumption, increased sequestration, or the quality of the transfused product was suboptimal. If there is minimal platelet production, but no accelerated platelet loss or sequestration, the recipient's platelet count should drop by approximately 33% each day following transfusion.³ When interpreting the success of a platelet transfusion, it should be noted that platelet counts at low values are imprecise.

Fresh Whole Blood

Component therapy is advocated, but FWB is the only platelet-rich product available to many veterinary clinics. It also has maximum hemostatic properties. In a human randomized clinical trial, 1 unit FWB was equivalent to 10 FWB-derived PCs after open heart surgery,³⁴ at least partially because the most functional platelets remain in the RBC fraction after centrifugation.³³ However, consistent with the observation that marked hemorrhage is required to lower platelet counts, so large-volume transfusions are required to substantially raise them. As a rule-of-thumb, a FWB transfusion of 10 mL/kg will raise the recipient's platelet count by a maximum of 10,000/ μ L and will stop critical hemorrhage if there is no ongoing platelet loss.² The transfusion is given over 1 hour unless a slower rate is indicated.

Platelet-rich Plasma and Platelet Concentrate

The recommended initial dose for FWB-derived PRP or PC is 1 unit/10 kg, although resources may dictate a smaller dose. If the units contain on average 6×10^{10} platelets, the 1-hour platelet increment should be $\sim 35,000/\mu$ L based on 51% PPR.⁴⁵ Assuming $\sim 50\%$ fixed platelet loss is perhaps extreme given that other studies suggest $\sim 20\%$ loss.^{6,49} However, in the author's experience platelet increments are even lower than that predicted with $\sim 50\%$ loss, perhaps reflecting decreased platelet survival in the presence of thrombocytopenia.^{25,29} Assuming canine apheresis PC contains on average 2.4×10^{11} in 200 mL (about four times the number of FWB-derived platelets), a minimum initial dose would be 1 apheresis PC/40 kg. Apheresis PCs are clearly superior for high-dose platelet support and platelet transfusion to large dogs. Cryopreserved apheresis PCs (Animal Blood Resources International) are available as half-units of 1.0×10^{11} platelets in 100 mL, with a recommended dose of 1 PC/10 kg and an

expected 1–2 hour increment of 20,000/ μ L, about 50% of that expected with FWB-derived PC.^{22,23} Fresh or cryopreserved PCs are transfused over 1 hour and PRP at 10 mL/kg/hour, unless a slower rate is indicated.

A vial of lyophilized apheresis PC (Animal Blood Resources International) contains approximately 5×10^{10} platelets, which are rehydrated in 60 mL of 0.9% sodium chloride just prior to administration; a recommended dose is 1 vial/15 kg administered over 15–20 minutes (A.S. Hale, personal communication).

Platelet-Poor Blood Components

If a platelet-rich product is not available, fresh-frozen plasma (10 mL/kg) or cryoprecipitate (1 unit/10 kg) could be considered as an alternative in the situation of severe hemorrhage due to thrombocytopenia. These products contain hemostatically functional platelet microparticles, and have been beneficial in treating thrombocytopenic bleeding.²¹ Hemostatic benefit may also be via augmentation of other aspects of hemostasis. Stored whole blood or packed RBCs may also reduce thrombocytopenic bleeding in anemic patients, as bleeding time is longer at lower hematocrits⁵⁰ (see Chapter 95): this is likely a benefit in dogs with IMT.

PLATELET TRANSFUSION REACTIONS

Transfusion reactions are discussed in Chapter 100. Febrile reactions are the most common reaction to PC; frequency is reduced by pre-storage leukoreduction.³⁸ Alloimmunization to platelet antigens (see Chapter 93) occurs readily in immunocompetent dogs receiving multiple transfusions, resulting in platelet transfusion refractoriness, a failure to achieve an expected increment in platelet count in response to a platelet transfusion.⁴⁵ Two currently accepted methods for preventing the development of platelet alloimmunization include leukocyte reduction and ultraviolet B irradiation.⁴³ The combination of centrifuge- and filter-leukoreduction was shown to be superior to either leukoreduction procedure alone for prevention of platelet alloimmunization in dogs.⁴³

Transfusion-associated sepsis is a particular concern with transfusion of PRP or PC due to the increased risk for bacterial proliferation at RT storage. The rate of contamination in human PC is 1 in 2000–5000 units.³¹ Visual inspection of a contaminated unit may reveal gaseous distention of the bag and loss of swirling, but contaminated units may have a normal appearance. Veterinary blood banks should closely monitor RT-stored platelet products microbiologically.

GRANULOCYTE TRANSFUSION

Granulocyte concentrate (GC) transfusion produces a transient increase in neutrophil numbers to a critical level that may permit survival in otherwise fatal sepsis. While promoted in the 1970s and early 1980s in human

medicine, usage of GC then declined because of expense, febrile reactions, controversial benefit, and newer antibiotics. The availability of granulocyte colony-stimulating factor (G-CSF) paradoxically led to a resurgence of GC transfusion.¹⁶ Granulocyte concentrate is usually prepared by continuous-flow centrifugation leukapheresis, with pre-administration of G-CSF (with or without corticosteroids) to the donor to increase neutrophil yield. Donors should be blood-type compatible, and irradiation of GC (25–50 Gy) is recommended to minimize transfusion-associated graft-versus-host disease.¹⁰

Therapeutic GC transfusions in small animals have been used primarily in experimental models of myelosuppression and neonatal sepsis.^{14,19} They have been rarely used in clinical veterinary medicine. In addition to leukapheresis, centrifugation of FWB, with or without colloid-facilitated sedimentation, may be used to isolate canine and feline buffy coats;^{7,41} sedimentation alone may also be used in the cat. Granulocytes are stored at RT without agitation for 24 hours.¹⁰ The initial dose is 1×10^9 granulocytes/kg in a volume of 15 mL/kg once to twice daily.

Granulocytes may also be used diagnostically. Infusion of radiolabeled autologous granulocytes and scintigraphy have been used to detect sites of inflammation in animals with nonspecific clinical signs.^{18,26}

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Blood Transfusion in Large Animals

MARGARET C. MUDGE

Indications for Blood Product Transfusions

- Whole Blood
- Packed Red Blood Cells
- Plasma
- Oxyglobin
- Blood Donor Selection
- Blood Typing and Crossmatching

Collection Techniques

- Administration and Adverse Reactions
- Ruminants and Camelids
 - Indications for Transfusion
 - Donor Selection
 - Collection Technique
 - Administration and Adverse Reactions

Acronyms and Abbreviations

CPDA-1, citrate-phosphate-dextrose with adenine; FFP, fresh frozen plasma; FPT, failure of transfer of passive immunity; NI, neonatal isoerythrolysis; PCV, packed cell volume; pRBC, packed red blood cell; RBC, red blood cell; TP, total protein; USDA, United States Department of Agriculture; WB, whole blood.

Blood product transfusion is an integral part of equine practice, both in referral institutions and in ambulatory practice. Blood products may be administered for conditions ranging from life-threatening acute hemorrhage to failure of transfer of passive immunity (FPT). The available equine blood products and indications for transfusion will be discussed in this chapter. Practical considerations for donor selection and collection and administration of blood will also be provided. A brief discussion of whole blood (WB) and blood product transfusion in ruminants and camelids is presented at the end of the chapter.

INDICATIONS FOR BLOOD PRODUCT TRANSFUSIONS

Whole blood

Whole blood transfusions are most often indicated for horses that have suffered acute blood loss from trauma, surgery, or other conditions such as splenic rupture or uterine artery hemorrhage. In cases of blood loss, the transfusion serves to restore blood volume as well as oxygen-carrying capacity. While there are no set variables that serve as “transfusion triggers,” a combination of physical examination and clinicopathologic param-

eters can be used to guide the decision to transfuse. It is important to remember that the packed cell volume (PCV) may remain normal for up to 12 hours in cases of acute hemorrhage due to the time required for fluid redistribution and the effects of splenic contraction. Serial monitoring of PCV and total protein (TP) as the horse is rehydrated with intravenous fluids will give an indication of the extent of blood loss. Suspicion of large volume blood loss, combined with tachycardia, tachypnea, pale mucous membranes, lethargy, and decreasing TP, may lead to the decision to transfuse.¹⁶ Blood transfusion is likely needed during an acute bleeding episode when the PCV drops below 20%, although in acute severe cases, transfusion may be needed before there is a significant drop in PCV.

Estimation of blood loss at surgery can be used to guide the decision to transfuse, with greater than 30% blood loss generally requiring transfusion.¹² Anesthetized horses may have very stable heart rate and PCV despite massive blood loss; pale mucous membranes with prolonged capillary refill time, decreasing TP, hypotension, and hypoxemia are better indicators of blood loss.⁴⁵

Oxygenation status can help to determine the need for blood transfusion in cases of both acute hemorrhage and chronic anemia. A rise in blood lactate concentration despite volume replacement with crystalloid or

colloid fluids may indicate continued tissue hypoxia and a need for blood transfusion.^{14,21} The oxygen extraction ratio is also a useful measure:

$$ER_{O_2} \approx \frac{S_{aO_2} - S_{vO_2}}{S_{aO_2}} \quad (\text{eqn 98.1})$$

where ER_{O_2} is the oxygen extraction ratio, S_{aO_2} the arterial oxygen saturation, and S_{vO_2} the mixed venous oxygen saturation. A ratio greater than 40–50% in the context of blood loss may indicate a need for blood transfusion.²²

Due to the short half-life of transfused red blood cells (RBCs), transfusion should be considered a temporary measure to restore oxygen-carrying capacity, relying on the horse's erythropoietic response or resolution of underlying disease to provide long-term resolution.^{19,36}

Packed Red Blood Cells

Packed RBCs (pRBCs) are indicated for normovolemic anemia, such as neonatal isoerythrolysis, erythropoietic failure, and chronic blood loss. In cases of chronic or hemolytic anemia, markers of tissue oxygenation, such as lactate and oxygen extraction are still useful. PCV is a better “transfusion trigger” for chronic anemia compared to acute hemorrhage, with transfusions suggested for horses with evidence of tissue hypoxia and a PCV less than 10–12%. Transfusions may be given at a higher PCV for horses with concurrent conditions (e.g. respiratory disease, anesthesia, sepsis) or risk of further blood loss. When pRBCs are not available, WB may be used for the same indications, although attention should be paid to the total volume given so that volume overload is avoided.

Plasma

Plasma transfusion is indicated for the treatment of clotting factor deficiency, hypoalbuminemia, decreased colloid oncotic pressure, and FPT. Fresh and fresh frozen plasma (FFP) contain immunoglobulins, coagulation factors (fibrinogen and factors II, VII, IX, X, XI, and XII), and cofactors (factors V and VIII), as well as the anticoagulant proteins antithrombin, protein C, and protein S. Plasma has also been used for treatment of disseminated intravascular coagulation in horses.⁴³

Colloid support is generally recommended in patients with a total protein less than 4.0 g/dL or serum albumin concentration less than 2.0 g/dL. Other indications for colloid support are colloid oncotic pressure less than 14 mmHg, clinical signs such as ventral edema, and conditions which increase microvascular permeability, such as sepsis. When plasma is not necessary for clotting factor replacement, a synthetic colloid such as hydroxyethyl starch (hetastarch) is preferred for volume expansion and more effective oncotic support.

TABLE 98.1 Commercial Sources of Fresh Frozen Equine Plasma

Veterinary Immunogenics (Cumbria, UK)	www.veterinaryimmunogenics.com
Lake Immunogenics, Inc. (Ontario, NY)	www.lakeimmunogenics.com
Mg Biologics (Ames, IA)	www.mgbiologics.com
EquiPlas (Templeton, CA)	www.plasvaccusa.com

Failure of transfer of passive immunity in neonatal foals more than 12 hours of age is best treated by plasma transfusion, as colostrum absorption is greatly diminished after 12 hours.¹⁸ An IgG concentration less than 200 mg/dL is considered complete FPT, and IgG between 400 and 800 mg/dL is considered partial FPT. Although plasma transfusion is not always needed for foals with partial FPT, it is recommended for foals that have pre-existing infection or exposure to pathogens. Commercially available fresh frozen hyperimmune plasma is most commonly used for treatment of neonatal foals (Table 98.1). Equine FFP is a USDA-licensed product, and most products have a minimum guarantee for IgG concentration and a 2–3 year shelf-life when frozen. Although commercially available hyperimmune plasma has very high IgG concentrations (1500–2500 mg/dL), plasma from local donor horses may provide better protection against specific local pathogens.

There are multiple hyperimmune plasma products with bacterial- or viral-specific antibodies. There is some evidence for the efficacy of *E. coli* (J5) and *Salmonella typhimurium* hyperimmune plasma for the treatment of equine endotoxemia; however, there are also reports which dispute the efficacy of such products.^{9,31,37,38} The use of *Rhodococcus equi* hyperimmune plasma for the prevention of *R. equi* has also been controversial.^{5,13,17,20,32} Other plasma products available for specific disease treatment include botulism antitoxin, West Nile virus antibody, and *Streptococcus equi* antibody.

Oxyglobin

Oxyglobin® (Biopure Corp., Boston, MA), a hemoglobin-based oxygen-carrying solution, has been used experimentally in ponies with normovolemic anemia.³ Oxyglobin improved hemodynamic and oxygen transport parameters; however, one pony did have an anaphylactic reaction. The use of Oxyglobin was also reported for treatment of a pony mare with chronic hemorrhage and a history of acute transfusion reactions.²⁵ Although Oxyglobin is currently commercially available, the cost and volume (125 mL) per bag limit its utility for equine treatment.

BLOOD DONOR SELECTION

The ideal equine blood donor is a healthy, young gelding weighing at least 500 kg. Donor horses should be up-to-date on vaccinations, including rhinopneumo-

nitis, tetanus, eastern and western encephalitis, rabies, and West Nile virus. Donors should be tested annually for equine infectious anemia. Because RBC antigens Aa and Qa are the most immunogenic, the ideal donor should lack the Aa and Qa alloantigens. There are breed-specific blood factor frequencies, so a donor of the same breed as the recipient may be preferable, especially when blood typing is not available. Horses that have received blood or plasma transfusions and mares that have had foals are not suitable as donors as they have a higher risk of carrying RBC alloantibodies. Donkeys have a RBC antigen known as “donkey factor,” which is not present in horses; therefore, donkeys or mules should not be used as donors for horses, as the transfused horses can develop anti-donkey factor antibodies.²⁶

When a surgical procedure is planned in advance and there is a high risk of blood loss, preoperative autologous donation should be considered, as the horse would be its own ideal blood donor.²⁸ The lifespan of allogeneic transfused RBCs is approximately 2–5 days, whereas autologous transfused RBCs have been shown to have a half-life of approximately 12 days.^{19,36} Intraoperative or post-hemorrhage cell salvage is also an option for autotransfusion, and its use has been reported in a horse with postcastration hemorrhage.³⁹ Red blood cell recovery can be performed with specialized cell salvage equipment which washes and filters collected blood, but cell salvage can also be performed with simple anticoagulation and filtration.⁴¹ The technique of cell salvage is limited to cases in which the salvaged blood is not in an area of infection or malignancy.

BLOOD TYPING AND CROSSMATCHING

In an emergency situation, an immediate blood transfusion may be given for the first time with a very minor risk of serious transfusion reaction. Horses can develop alloantibodies within 1 week of transfusion, so blood typing and crossmatching are recommended before a second transfusion is performed.⁴⁶ However, a second blood transfusion may be performed safely within 2–3 days of the first transfusion without a blood crossmatch.

Blood typing and alloantibody screening can be used to help find the most appropriate donor horse for the patient requiring transfusion (see Chapters 92 and 139). Unfortunately, since blood typing is time-consuming and laboratories performing blood typing are very limited, this is not often a practical method of donor selection (Table 98.2). Blood typing and antibody screening prior to initial transfusion are more important for horses for which subsequent blood transfusions are anticipated and for broodmares which may produce foals with neonatal isoerythrolysis (NI) if sensitized to other blood group factors.⁴⁶ A rapid agglutination method for detection of equine RBC antigens Ca and Aa has been developed which may be more practical for pretransfusion testing.³⁰

TABLE 98.2 Equine Blood Typing Laboratories

University of California, Davis Hematology Laboratory Room 1012, Veterinary Teaching Hospital One Garrod Drive University of California, Davis Davis, CA 95616 Phone: 530-752-1303
University of Kentucky Equine Parentage Testing and Research Lab 102 Animal Pathology Building Lexington, KY 40546-0076 Phone: 859-257-3656 www.ca.uky.edu/gluck/ServEPVL.asp
Rood and Riddle Veterinary Laboratory 2150 Georgetown Rd. Lexington, KY 40511 Phone: 859-233-0331 www.roodandriddle.com
Hagyard Equine Medical Institute 4250 Iron Works Pike Lexington, KY 40511-8412 Phone: 859-259-3685 www.hagyard.com

A blood crossmatch is recommended prior to a transfusion, especially for any horse which may have previously been exposed to RBC antigens (see Chapters 92 and 139). Hemagglutination crossmatching is widely available and rapidly performed; however, it will not predict all transfusion reactions, namely the hemolytic reactions. Rabbit complement can be added to the reaction mixture to detect hemolytic reactions.²

COLLECTION TECHNIQUES

Blood is collected from the jugular vein of the donor horse, either via direct needle cannulation or catheterization. When a large volume of blood is needed, a 10 or 12 gauge catheter is recommended, although a 14 gauge catheter is also sufficient. Blood flow may be improved by placing the catheter opposite the venous blood flow (catheter directed toward the head). A healthy horse can donate approximately 20% of its total blood volume every 30 days.²⁴ When 15% or greater blood volume is collected, volume replacement with intravenous crystalloid fluids is recommended. The donor horse's heart rate, respiratory rate, and attitude should be monitored during the blood collection. Vital parameters should normalize within 1 hour of collection.

Plastic bags and vacuum-collection glass bottles are available for blood collection in sizes ranging from 450mL to 2L (Baxter Fenwal, Deerfield, IL; MWI Veterinary Supply, Meridian, ID). The glass bottles are preferred by many due to the speed of collection; however, the glass inactivates platelets and causes some damage to RBCs.^{27,33}

When blood is collected for immediate transfusion, anticoagulation with 3.2% sodium citrate is adequate.

However, when blood is stored for later transfusion, optimal pH and support of RBC metabolism are necessary to sustain RBC viability (see Chapter 94). Although post-transfusion viability studies have not yet been performed on stored equine blood, biochemical and hematologic parameters suggest that WB may be stored in CPDA-1 bags for at least 3 weeks.²⁷ Red blood cell concentrates stored in saline-adenine-glucose-mannitol solution may be suitable for transfusion for up to 35 days after collection.²⁹ Storage conditions for equine blood are similar to those for canine and human blood (see Chapter 94).

Equine blood can be processed to provide plasma and pRBC components, as with other species. Due to the rapid sedimentation of equine RBCs, the RBC component can be administered without specialized processing; however, the pRBCs will still contain plasma components unless centrifugation and repeated washing are performed. Washing of RBCs is the preferred technique when a transfusion is given to an NI foal using the mare as a donor. When RBC washing or other processing is planned, blood should be collected into bags rather than bottles due to ease of centrifugation and sterile transfer.

Plasma processing can be performed by gravity sedimentation, centrifugation using a double-bag system, or plasmapheresis. Plasmapheresis is the preferred technique as it is more rapid than WB collection and processing, and results in plasma with minimal RBCs and leukocytes.¹⁰ Plasmapheresis of between 4 and 11 L can be performed every 30 days on donor horses.²³ Immunoglobulins are well-maintained for at least 1 year in FFP; however, coagulation factor activity may decrease after 2–4 months of storage.¹⁵

The process of plateletpheresis has not been described for the collection of equine platelets in the clinical setting. Platelet-rich plasma has been prepared from fresh WB in horses, especially for orthopedic applications such as injection of injured tendons.⁴⁰

ADMINISTRATION AND ADVERSE REACTIONS

The volume of blood to be transfused depends on estimated blood loss, estimated total blood volume, and donor PCV:

$$\begin{aligned} &\text{Blood transfusion volume (mL)} \\ &= \text{Body weight (kg)} \\ &\quad \times \text{Blood volume (mL/kg)} \\ &\quad \times \left[\frac{(\text{Desired PCV} - \text{Actual PCV})}{\text{Donor PCV}} \right] \quad (\text{eqn 98.2}) \end{aligned}$$

In cases of acute blood loss, PCV is often not useful for estimates of volume to be transfused since it does not accurately reflect blood loss. Instead, estimates of blood loss and evaluation of clinical parameters are used to determine the volume of blood needed. Between 25% and 50% of the total blood lost should be replaced

by transfusion since much of the circulating volume will be replaced by fluid shifts. It is important to remember that up to 75% of RBCs lost into a body cavity (e.g. hemoperitoneum) are autotransfused back into circulation within 24–72 hours.³⁴ Therefore, lower percentages of blood volume replacement may be needed in cases of intracavitary hemorrhage. Blood and plasma products should be delivered with an in-line filter to remove small clots and fibrin.

Volumes of plasma for treatment of hypoproteinemia can be estimated by TP or albumin concentrations, although the use of plasma to normalize severe hypoproteinemia can be prohibitively expensive in the adult horse.

$$\begin{aligned} &\text{Plasma transfusion volume (mL)} \\ &= \text{Body weight (kg)} \times 45 (\text{mL/kg}) \\ &\quad \times \left[\frac{(\text{Desired TP} - \text{Actual TP})}{\text{Donor TP}} \right] \quad (\text{eqn 98.3}) \end{aligned}$$

Volumes of plasma given for treatment of hypoproteinemia or coagulopathy are often determined by clinical and clinicopathological response. The volume of plasma needed in a foal with FPT can be determined if the IgG concentrations of the foal and the plasma are known. A dose of 20 mL/kg of plasma (IgG approximately 1200 mg/dL) will generally raise the foal's IgG concentration by 200–300 mg/dL. A larger volume of plasma may be needed to achieve a similar rise in IgG in clinically ill foals.⁴⁴

In order to monitor for transfusion reactions, blood should be delivered at a rate of approximately 0.3 mL/kg over the first 10–20 minutes, while monitoring heart rate, body temperature, and respiratory rate. Horses should also be monitored for signs of muscle fasciculation, piloerection, and urticaria. Adverse reactions reported in horses receiving blood transfusions include urticaria, hemolysis, and acute anaphylactic reactions. The rate of adverse reaction to WB transfusion has been reported as 16%, with 1 of 44 horses (2%) having a fatal anaphylactic reaction.¹⁶ If no signs of reaction are seen, the rate of administration can be increased to 5 mL/kg/h for normovolemic horses and up to 20–40 mL/kg/h for hypovolemic horses. If signs of anaphylaxis are present, epinephrine (0.01–0.02 mL/kg IV of 1:1000 solution) should be administered immediately. More mild transfusion reactions, such as urticaria, fever, and tachypnea, may be treated with an anti-inflammatory (e.g. flunixin meglumine 1.1 mg/kg IV) or an antihistamine (e.g. tripeleminamine 1.1 mg/kg IM).

Similar to the risks in other veterinary species, bacterial contamination of blood, transmission of blood-borne disease from donor to recipient, and hypocalcemia associated with citrate toxicity are all potential concerns related to transfusion in the equine patient (see Chapter 100). An additional concern in horses is the possible sensitization of a broodmare to blood group antigens, leading to the risk of NI in subsequent foals.⁴⁶ Although plasma transfusions are not commonly associated with serious adverse reactions, serum hepatitis has been

reported in association with transfusions of commercial plasma.¹

RUMINANTS AND CAMELIDS

Indications for Transfusion

The indications for WB and plasma transfusion in ruminants and camelids are similar to those for horses. Chronic anemia may be a more common problem in ruminants, as gastrointestinal parasites, especially *Haemonchus contortus*, and ectoparasites (e.g. *Haematopinus* spp. and *Linognathus* spp.) are causes of chronic blood loss anemia, and iron-deficiency anemia can affect neonatal calves.³⁵ The blood parasites *Anaplasma* and *Babesia* are both associated with the development of chronic anemia although by different mechanisms. Infections in llamas and alpacas due to *Mycoplasma haemollama* result in a chronic anemia due to extravascular hemolysis, frequently resulting in a PCV of less than 10%. In the case of acute hemorrhage or ongoing blood loss (e.g. abomasal ulcers), transfusion should be considered when the PCV falls below 20%. Ruminants with chronic blood loss may not require blood transfusion until the PCV is below 12%.

Failure of transfer of passive immunity is a common indication for plasma transfusion in neonatal calves and crias. While a TP less than 5.0 g/dL is suggestive of FPT in neonates, radial immunodiffusion can be performed to quantitatively determine IgG concentration.^{4,42} Commercial FFP is available for both ruminant and camelid transfusion (Animal Blood Resources International, Stockbridge, MI), although fresh plasma or even WB can be very effective for treatment of FPT. Hyperimmune serum products are available for subcutaneous and intramuscular dosing in ruminants, including products with antibodies against *E. coli*, *Pasturella*, *Aerobacter pyogenes*, *Salmonella typhimurium* and *Clostridium* (Poly Serum, Novartis Animal Health, Greensboro, NC). The use of Oxyglobin has been reported in a sheep with anemia due to gastrointestinal parasites and in two alpacas with hemolytic anemia; however, Oxyglobin is not commonly used in larger animals due to cost and more ready availability of WB from donors.^{7,11}

Donor Selection

The initial blood transfusion should generally be safe, regardless of the donor; however, a J-negative donor (cattle) is ideal (see Chapter 92).⁸ Routine crossmatching is not useful in ruminants since agglutination reactions do not occur. Testing for hemolytic reactions should be performed using complement, although this testing is not available in all laboratories. Initial transfusions are generally safe to administer without a blood crossmatch. However, if more than 48–72 hours have elapsed since the initial blood transfusion, crossmatching is recommended. Blood donors should be free of disease, including bovine leukosis virus, anaplasmosis, and

bovine viral diarrhea virus. If a donor animal is selected from the herd, it is especially important to check PCV and TP prior to blood collection, since the donor animal may also be affected by the same endo- or ecto-parasites.

Collection Technique

Up to 20–25% of total blood volume can be removed from the donor animal, usually via needle cannulation or jugular catheterization, with total blood volume estimated at 80 mL/kg in cattle. As with equine transfusions, blood can be collected into bottles or bags with citrate anticoagulant. Blood stored in CPDA-1 may retain adequate viability for 2–3 weeks.⁶ Since bovine blood does not readily separate, a centrifuge is needed to process WB into plasma and pRBC components.

Administration and Adverse Reactions

Blood should initially be delivered slowly in order to monitor for transfusion reactions. Complement-mediated hemolysis is the primary transfusion reaction which occurs with ruminant blood type incompatibility. Volume overload should be avoided, and total volumes delivered should especially be considered in neonates and small ruminants.

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Blood Transfusion in Exotic Species

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Acronyms and Abbreviations

ACD, acid-citrate-dextrose; CPD, citrate-phosphate-dextrose; CPDA-1, citrate-phosphate-dextrose-adenine; CRI, constant rate infusion; Hgb, hemoglobin; HBOC, hemoglobin-based oxygen carrier; IMHA, immune-mediated hemolytic anemia; IO, intraosseous; PCV, packed cell volume; RBC, red blood cell; WB, whole blood.

Transfusion medicine in exotic species is both exciting and frustrating to the clinician. Often, information from more traditional companion species is extrapolated to guide the treatment of exotic species. There is still much anecdotal information on the subject of transfusion medicine; however, experimental data will be provided when possible.

AVIAN

Anemia in Birds

Anemia in birds is caused by the same mechanisms as in mammals: blood loss, hemolysis, or ineffective erythropoiesis. Blood loss anemias are most commonly seen in birds secondary to injuries, although more chronic forms, such as gastrointestinal bleeding, can occur. Healthy birds are remarkably adept at dealing with acute blood loss. Clinical signs of anemia and hypovolemia do not usually occur until 50–60% of the blood volume is lost.¹⁵ Hemolytic anemias are less common in birds but can be caused by toxicities, hemoparasites, and septicemia. Immune-mediated hemolytic anemia

(IMHA) has recently been reported in a pet bird.¹⁴ Ineffective erythropoiesis is often seen as an anemia of chronic disease associated with underlying infectious, metabolic, and neoplastic disorders. Birds develop this type of anemia more quickly than mammals because of the shorter lifespan of the avian red blood cell (RBC).

Indications for Transfusion

The decision to administer a blood transfusion is based on several factors: packed cell volume (PCV), clinical condition of the patient, chronicity of the anemia, cause and severity of the anemia, possibility of further blood loss (by either hemorrhage or diagnostic sampling), and the ability of the patient to tolerate the stress associated with administering a transfusion. A study of acute blood loss in pigeons demonstrated that birds receiving intravenous crystalloid fluids alone had the best response to hemorrhage, as judged by the PCV at 24 and 48 hours, when compared to birds receiving heterologous (different species) and homologous (same species) transfusions.⁴ However, another study in pigeons in which 60% of the blood volume was removed

showed that the homologous transfusion of 30% of the blood volume ameliorated the effects of the anemia, but both birds with and without a transfusion returned to a normal PCV by day 6.¹⁰ These findings suggest that the best course of action in acute blood loss in birds would be intravenous administration of balanced crystalloids for mild to moderate hemorrhage (loss of up to 25% of the blood volume) and a blood transfusion for moderate to severe hemorrhage (greater than 25% loss of blood volume).

There is evidence that birds may be better able to deal with chronic anemias because the nucleated RBC can produce more hemoglobin (Hgb) in response to anemia.²⁷ Anemias of a chronic nature, therefore, may not require transfusions if the patient is stable and no further blood loss is expected. Clinical evidence, however, suggests that birds may benefit from transfusions even with chronic anemia because of increased oxygenation of tissues and improved healing and appetite.

Blood Groups

Blood group antigens have been studied in chickens and other species in an attempt to identify gene markers associated with desirable traits and for pedigree purposes.¹¹ Twenty-eight blood group antigens have been found in the chicken, many of which have more than 10 alleles for a single antigen.⁹ To date, there is no available information regarding blood groups in pet birds.¹³ Serologic incompatibility has been demonstrated in a number of parrot species. All blood crossmatches between African grey parrots to cockatiels were incompatible, as were most crossmatches between umbrella cockatoos to cockatiels and pigeons to cockatiels. Crossmatch reactions were found in one study between

various species of conures, yet administration of these apparently incompatible transfusions resulted in an increase in the PCV and no untoward effect on the recipient.⁷

Compatibility and Donor Selection

Because of the lack of identified blood groups in pet birds, compatibility for transfusion is based on blood crossmatching (see Chapter 139). Several studies have shown homologous transfusions to have a longer RBC survival time when compared to heterologous transfusions.^{4,6,24} Heterologous transfusions between members of the same genus may have similar survival times as homologous transfusions.^{2,12} Therefore, the recommended donor is a bird of the same species or, if not possible, the same genus.

Unlike mammals, a single transfusion from different species may be safe and efficacious.^{2,12} The half-life of heterologous transfusions, however, ranges between 12 hours and 3 days, much shorter than that of a homologous transfusion which lasts for 6–11 days.^{6,24} With the number of avian species seen in practice, keeping a large variety of blood donors is impractical. A feasible option is to have a list of client-owned, healthy birds of a variety of species to use as donors when necessary. Donor birds should be screened as in Table 99.1.

An alternative to whole blood (WB) transfusions is the use of an Hgb-based oxygen carrier (HBOC), such as Oxyglobin® (Biopure Corp., Cambridge, MA), an ultrapurified polymerized bovine Hgb solution. HBOCs eliminate the need for crossmatching and can be used as both a colloid and RBC substitute in any species of bird. Following Oxyglobin administration to chickens, mild renal tubular damage was evident in some birds

TABLE 99.1 Donor selection criteria for exotic species

Species	Ideal Donor	Donor Screening
Avian (psittacine)	The same species or genus Housed singly or closed aviary One time heterologous transfusion possible	Yearly physical examination, CBC and biochemical profiles Screened for polyoma, psittacine beak and feather disease, and chlamydiae Major and minor crossmatch
Ferret	Male, less than 3 years of age	Yearly examination, CBC, heartworm testing (antibody and antigen) and biochemical profiles Placed on heartworm preventatives in endemic areas Vaccinated yearly for canine distemper and rabies
Rabbit	Female, less than 3 years of age Related animals may reduce disease transmission	Yearly examination, CBC, and biochemical profile Negative testing for <i>Encephalitozoon cuniculi</i> Screened for viral hemorrhagic disease and myxomatosis (in endemic countries) Major crossmatch
Rodents	Male guinea pigs less than 3 years of age Female chinchillas less than 5 years of age Male rats less than 1 year of age	Yearly examination, CBC and biochemical profile in larger species Major crossmatch
Reptiles	Young, healthy animal of same species Same genus Singly housed or closed herpetarium Do not use blood filter	Yearly examination, CBC and biochemical profile PCV, blood glucose and total solids in smaller species Screen for hemoparasites

at 24–48 hours, but these changes were subclinical and not detected at day 7.¹

Blood Collection and Storage

In general, it is safe to withdraw 10–20% of the blood volume of a healthy bird (with a standard blood volume of 100 mL/kg this would be approximately 1–2 mL/100 g body weight). For psittacines, ratites and soft bills, the right jugular is usually the most reliable and readily accessible site for blood collection. In waterfowl and gallinaceous birds, the medial metatarsal vein is easily identified on the dorsomedial surface of the lower leg and can be used, in addition to the jugular vein, for blood collection. The basilica or wing vein can be visualized as it crosses the proximal ulna and can be used in most species, especially pigeons and doves, but may be smaller and more fragile than other veins.

Blood may be collected into syringes containing an anticoagulant, preferably 3.2% sodium citrate. Heparin, acid-citrate-dextrose (ACD), citrate-phosphate-dextrose (CPD), or CPD with adenine (CPDA-1) can also be used. Sodium citrate is added to the syringe before collection of the blood at 0.1 mL of citrate per 0.9 mL of blood. A similar volume of CPD, CPDA-1, and ACD can be used. Care should be taken when transfusing smaller species or hypocalcemic patients since citrate binds calcium in the blood. Alternatively, heparin can be used for avian transfusions at 0.25 mL per 10 mL of blood collected.

Blood is collected into a syringe using a butterfly catheter or injection needle. A small amount of anticoagulant should be flushed through the needle to prevent clotting in the needle. The sample may then be administered from the collection syringe. The author has used WB stored in ACD for 24 hours without apparent deleterious effect; however, the long term storage of avian blood in this medium is not possible because of differences in avian RBC metabolism.¹⁹

Blood Administration

The amount of WB administered will depend on several factors, including the degree of anemia, size of the donor, and the stability of the patient. As a general guideline, 10–20% of the blood volume of the recipient (1–2 mL/100 g) is administered in most circumstances. If more is needed, several donors may be used. Transfusions can be given IV or IO (intraosseous), as a bolus or by constant rate infusion (CRI). Constant rate infusion is preferred because it is less likely to cause circulatory overload but does require the placement of an indwelling IV or IO catheter. Indwelling IV catheters can be placed in the median metatarsal vein in larger psittacines and all fowl; IO catheters are placed in the distal ulna or proximal tibiotarsus. The transfusion should be given over 2–4 hours, depending on how quickly the blood was lost. A blood filter should be used to remove clots or cellular aggregates from the transfusion.

The bolus method of transfusion delivery has the advantage of requiring only temporary venous access

but can cause circulatory overload and may require longer handling time. In some patients, it can be useful to use a butterfly catheter to obtain blood for analysis and then deliver a transfusion and other IV treatments using the same catheter. A bolus transfusion is typically given over 1–5 minutes, quickly enough to avoid over-stressing the patient but slowly enough to prevent circulatory overload.

Transfusion Reactions

Acute hemolytic reactions have not been reported in birds receiving a single blood transfusion. Incompatible blood crossmatches, however, have been reported.^{6–8,24} In one study 66% of heterologous crossmatches in raptors, pigeons, parrots, and gallinaceous birds showed agglutination and hemolysis.²⁶ In another study of psittacine crossmatches, all major crossmatches between African gray parrots (*Psittacus erithacus*) and cockatiels (*Nymphicus hollandicus*) were found to be incompatible, and 80% of the cockatoo (*Cacatua alba*) to cockatiel crossmatches were incompatible.⁸ In addition, all blue-fronted Amazon parrots (*Amazona aestiva*) were found to be compatible on the first transfusion, but four of five pairs were incompatible before the second transfusion.⁸ Interestingly, no visible signs of transfusion reactions were seen in any of the recipient birds, and all birds appeared healthy while monitored for 6 months after three separate transfusions.

Transfusion reactions have been reported in two other studies in birds given multiple heterologous transfusions.^{2,12} These birds were given three transfusions at 2-week intervals, resulting in the deaths of five of six birds. Histopathology revealed hemoglobin casts in the renal tubules, consistent with hemolytic transfusion reactions. Antibodies against heterologous RBCs lasted approximately 3 weeks in one group of birds studied.

FERRETS

The most unique aspect of transfusion medicine in ferrets is the lack of detectable blood groups.¹⁷ No naturally occurring anti-RBC antibodies were identified when serum and RBCs of 26 ferrets were randomly paired and tested for agglutination and hemolysis. Clinically, ferrets have received multiple blood transfusions without developing transfusion reactions.¹⁸ In light of this information, blood crossmatching ferrets before transfusion is not standard protocol.

Anemia in Ferrets

Blood loss may be caused by trauma, severe flea infestation, and gastrointestinal bleeding secondary to foreign bodies, gastric ulcers, helicobacteriosis, and gastroenteritis. These conditions can result in severe hemorrhage that warrants a transfusion as part of the supportive care regimen. IMHA has not yet been reported in this species.

Ineffective erythropoiesis in ferrets has been reported with a variety of conditions, including lymphoma and other neoplasias, hyperestrogenism, Aleutian disease, adrenal disease, and anemia of chronic inflammation.^{3,20,23,25} In addition, the author has diagnosed anemia due to presumptive immune-mediated erythroid hypoplasia in a ferret that responded to immunosuppressive doses of prednisolone.

Indications for Transfusion

As in other species, the decision to administer a transfusion should be based on the PCV and the clinical status of the ferret. Transfusions in ferrets are generally recommended if the PCV falls below 25%, more than 30% of the blood volume is lost (blood volume is approximately 8% of the body weight), blood loss is associated with collapse, or ongoing hemorrhage is present.^{22,25} Ferrets may tolerate PCVs lower than 25% if the anemia is of gradual onset. The blood volume required for transfusion can be estimated using the same formulas for other mammals. In general, the administration of 20mL/kg of WB will increase the PCV by approximately 10%.

Donor Selection and Blood Collection

Healthy male ferrets are suggested for blood donors because of their larger size and blood volume. The donors should be screened as in Table 99.1.

An alternative to WB transfusion in ferrets is the use of a HBOC. Oxyglobin has been used with success in ferrets, both as a RBC substitute and a colloid. A suggested dose is 6–15mL/kg IV administered over 4 hours.²¹

Blood for transfusion is typically collected from the donor using inhalant anesthesia or other sedation. Isoflurane anesthesia has been shown to decrease PCV, Hgb concentration, and RBC count in ferrets as soon as 15 minutes after induction.¹⁸ Blood should be collected as quickly as possible if using isoflurane. Sevoflurane has not been tested but is thought to cause similar splenic sequestration of RBCs in ferrets. Tractable ferrets may be sedated sufficiently using butorphanol at 0.3–0.5mg/kg IM. Propofol (2–5mg/kg) may be given via an IV catheter or butterfly catheter in the jugular vein or cranial vena cava and will cause rapid anesthesia and recovery in the donor ferret.

Blood for transfusion is best collected from the cranial vena cava or jugular vein using 23–25 gauge butterfly catheters. Jugular venipuncture is similar to other species. The cranial vena cava or base of the jugular vein is accessed at the thoracic inlet. The needle is inserted at the angle between the manubrium and base of the first rib and directed caudally toward the opposite hind leg. The blood is collected into an appropriately sized syringe containing 3.2% sodium citrate, ACD or CPDA-1. For citrate and ACD, the ratio of anticoagulant to blood is 1:7, whereas for CPDA-1 the ratio is 1:9. Long-term storage of ferret WB is possible using ACD or CPDA-1, but is not currently practised.

Blood Administration

The blood should be administered by syringe pump via CRI over a 4 hour period. The blood can be administered into a peripheral or jugular venous catheter. Intravenous catheters (24–26 gauge) can be placed in the cephalic or lateral saphenous veins. A cut-down procedure may be required to place a jugular catheter. Intraosseous catheters can be placed in the proximal femur or tibial crest in small patients or in patients with vascular compromise. A blood filter should be used to prevent the transfusion of clots or larger cellular aggregates.

Transfusion Reactions

Transfusion reactions have not been reported in ferrets, ostensibly because of the lack of detectable blood groups due to an absence of genetic diversity, as in cheetahs. Ferrets have received up to 13 separate transfusions from many different ferrets without apparent development of anti-RBC antibodies.²³

RABBITS AND RODENTS

There is limited experimental work on transfusions in rabbits and rodents, but the author has administered many transfusions to rabbits, guinea pigs, chinchillas, and rats with success.

Anemia in rabbits and rodents is caused by similar mechanisms as in other species. Blood loss can occur from traumatic injuries, as well as reproductive problems such as uterine aneurysms, adenocarcinomas, endometrosis, and dystocia. Hemolytic anemias are uncommon in these species but can occur with toxin ingestion. Anemia of chronic disease is a common finding in sick rabbits and rodents. A PCV of 30% (reference interval, 35–48%) is a common finding in animals with chronic illness.

Indications for Transfusion

A transfusion is indicated in rabbits and rodents with a PCV less than 20% or in animals experiencing hypovolemic shock from acute blood loss. Some authors recommend transfusions at lower PCVs in rabbits, such as 10–15%.⁵ The duration of anemia and clinical signs will also help to determine at which point a transfusion is given. Acute blood loss of 20–25% can result in shock in this species and warrants a transfusion using WB or an HBOC as part of the treatment protocol.

Donor Selection and Blood Collection

Blood donor criteria are presented in Table 99.1. In rabbits blood can be collected from the jugular vein, central artery of the ear, or lateral saphenous vein. Severe damage to the ear artery can result in loss of the pinna, so owner consent to this approach is mandatory. Most rabbits will have to be lightly sedated for collec-

tion of blood for a transfusion. The use of topical anesthetic cream (2.5% lidocaine and 2.5% prilocaine, EMLA, AstraZeneca, Wilmington, DE) is helpful. The author routinely uses midazolam (1 mg/kg IM) and butorphanol (0.5 mg/kg IM) for this procedure. Inhalant anesthesia can also be used. A volume of 1% of the body weight can be safely collected from the donor. Blood should be collected using 21–25 gauge butterfly catheters and the standard anticoagulant/preservative solutions as mentioned above. Whole blood may be stored at 4–6°C in CPD-A for 28–35 days.⁵

In guinea pigs and chinchillas the blood is usually collected from the jugular vein or cranial vena cava. Donors are sedated using inhalant anesthetics or the midazolam/butorphanol combination described above. Total blood volume of the donor is approximately 70 mL/kg, and it is considered safe to remove 10% of this volume. Blood is collected via 25 gauge butterfly catheters using the standard anticoagulant/preservative solutions. There is no information on storage of rodent blood.

Blood Administration

Information on blood types in rabbits and rodents is not readily accessible. Therefore, at a minimum, a major crossmatch is warranted in these species. Transfusions should ideally be given over 4 hours; however, faster delivery is warranted in acute blood loss. A syringe pump can be used with a low flow extension set and blood filter. Intravenous catheters can be placed in the lateral saphenous and cephalic veins of rabbits, guinea pigs, and chinchillas, and in the lateral tail vein of rats. In some cases it is difficult to place an indwelling catheter, so transfusions can be given into the vena cava in rodents or lateral ear vein or lateral saphenous in rabbits using a butterfly catheter. Bolus transfusions should be given over 5 minutes, if restraint for that length of time is possible, in an effort to avoid circulatory overload of the recipient.

Transfusion Reactions

Acute hemolytic transfusion reactions have not been documented in rabbits or rodents. However, it has been suggested to give the transfusion slowly (0.25 mL/kg/h) for the first 15 minutes to detect any potential adverse reactions, such as urticaria, anaphylaxis, erythema, or fever.⁵

GUIDELINES FOR OTHER SPECIES

There are several guidelines to follow when considering a transfusion in other species. In general, a transfusion is warranted in acute blood loss of greater than 30% of an animal's blood volume or a 50% decrease in the low normal PCV. In reptiles, transfusions should be considered when the PCV is 15% or less. The clinical assessment of the animal is crucial. If the patient is tolerating an anemia, the risks and benefits of a transfusion should

be considered. The clinical condition of most animals improves dramatically with a transfusion of even 10% of the blood volume from a healthy donor. The donor should be a large, healthy specimen of the same species, although the author has used the same genus in reptiles with success. At a minimum, a major blood crossmatch should be performed to help determine the potential for transfusion reactions. A new donor should be found if an incompatibility is noted on the blood crossmatch. If this is not possible, the clinician may consider administration of an HBOC. The blood transfusion should be given over 2–4 hours, and the animal monitored for signs of a transfusion reaction, as above. If an adverse reaction is noted, the transfusion should be discontinued immediately and the recipient monitored for signs of an acute hemolytic transfusion reaction (e.g. hemoglobinemia, hemoglobinuria, lack of rise in PCV). A blood filter is useful in most species but it has been recommended that a blood filter not be used in reptiles because of the large RBC size causing damage to or limiting the passage of cells.¹⁶

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Transfusion Reactions

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Immune Complications

Hemolytic Transfusion Reactions

- Acute hemolytic transfusion reactions
- Delayed hemolytic transfusion reactions

Non-hemolytic Transfusion Reactions

- Febrile and allergic reactions
- Uncommon immune reactions

Non-Immune Complications

Infectious Disease Transmission

Transfusion-associated Sepsis

Uncommon Non-Immune Complications

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- Circulatory overload
- Non-immune hemolysis unrelated to bacterial contamination

- Prevention and Recognition of Transfusion Reactions
- Evaluation of a Patient with a Suspected Transfusion Reaction

Acronyms and Abbreviations

AHTR, acute hemolytic transfusion reaction; DHTR, delayed hemolytic transfusion reaction; FFP, fresh frozen plasma; FNHTR, febrile non-hemolytic transfusion reaction; HTR, hemolytic transfusion reaction; PCV, packed cell volume; pRBCs, packed red blood cells; PTP, post-transfusion purpura; RBC, red blood cell; TA-GVHD, transfusion-associated graft-versus-host-disease; TRALI, transfusion-related acute lung injury; WB, whole blood.

Blood is a complex biologic product which, like any tissue transplant, can result in as much harm as benefit to the recipient. The term transfusion reaction denotes any adverse event associated with transfusion of blood or a blood component. The reaction may occur during or within hours to weeks after administration of a blood product. Understanding how to prevent a transfusion reaction and recognizing the potential clinical and clinicopathologic signs of an adverse reaction should one occur are key to good transfusion medicine practice.

Transfusion reactions have been classified using various terminologies in both the human and veterinary literature but are most often characterized by immunologic and non-immunologic mechanisms. There have been several reviews in the veterinary literature on blood transfusions in cats, dogs, and horses, with the incidence and type of transfusion reactions varying between institutions and species but ranging from 3–13%.^{10,11,19,20,23–26} Reports of adverse reactions underscore the need for further education on their prevention and identification.

IMMUNE COMPLICATIONS

Hemolytic Transfusion Reactions

Hemolytic transfusion reactions (HTR) jeopardize the health of an already compromised patient. A HTR is the result of naturally-occurring or induced antibodies present in the recipient plasma which can destroy donor red blood cells (RBCs) immediately upon transfusion or within hours to weeks. These reactions are classified as a type 2 hypersensitivity and are mediated by antibodies directed against antigens present on the surface of RBCs.¹ After reacting with the RBC surface antigen, IgG or IgM antibodies activate the complement system, culminating in the formation of a membrane attack complex which disrupts the lipid bilayer of the RBC membrane, causing intravascular hemolysis.¹ Also, antibody or complement fragments, primarily C3b or C4b, can adhere to the RBC surface (opsonization), enhancing RBC susceptibility to phagocytosis by leukocytes expressing receptors for these proteins.¹ Either intravascular or extravascular hemolysis can result, depending

on whether the leukocytes recognize the opsonized RBCs while in circulation or within the reticuloendothelial system. The severity of a HTR is influenced by the concentration of recipient alloantibody, the immunoglobulin type, whether the antibody is warm or cold-reacting, the amount of blood transfused, and the condition of the recipient.⁴⁶

Acute Hemolytic Transfusion Reactions

Acute hemolytic transfusion reactions (AHTRs) present within 24 hours of a RBC transfusion.^{12,36} Preformed antibodies in the recipient react with transfused donor RBCs. The recipient has previously formed an alloantibody against an antigen present on the donor's RBCs which is not present on the recipient's RBCs. Cats are similar to humans, as both have naturally-occurring alloantibodies which can result in a potentially fatal AHTR. Anti-A alloantibodies in type B cats are strong hemagglutinins and hemolysins and are present in a high concentration^{8,28} (see Chapter 92). Transfusion of type A blood to a type B cat can be fatal if not immediately recognized, with subsequent discontinuation of the transfusion and institution of supportive care.^{11,15,16} Within seconds to a few minutes of receiving as little as 1 mL of type A blood, a type B cat may exhibit restlessness, vocalization, vomiting, urination, salivation, and recumbency.^{15,16} Bradycardia, cardiac arrhythmias, decreased pulse strength, pale mucous membranes, and prolonged capillary refill time are also expected in the initial phase.¹⁶ If the reaction is recognized and the transfusion stopped, hemoglobinemia and hemoglobinuria will follow, as these reactions are IgM and complement-mediated with obvious intravascular hemolysis.^{15,16} Acute renal failure, which is seen in humans with blood type ABO transfusion reactions, has not been documented in type B cats receiving type A blood under experimental conditions.

As type B blood is not commonly stored and type B cats comprise a small percentage of cats (outside of a few breeds), inadvertent transfusion of type B blood to a type A cat is unlikely. Inaccurate blood typing results, however, could result in a type A or AB cat being mislabeled as type B with subsequent transfusion of type B blood.³³ Although type A cats generally have lower concentration of weak anti-B antibodies which are of both IgM and IgG classes, transfusion of type B blood can still cause both clinical and clinicopathologic features of a transfusion reaction including discomfort, listlessness, tachycardia and tachypnea, as well as mild hemoglobinemia, hemoglobinuria, and bilirubinuria.^{8,16,28} Direct antiglobulin tests (Coombs' test) are variably positive in both type A and type B cats receiving mismatched blood.¹⁶

A blood type AB cat could receive either type A or B pRBCs, but plasma or whole blood (WB) from a blood type A or B cat could increase the risk of a HTR reaction due to the presence of anti-B and anti-A alloantibodies, respectively. Given the generally weaker nature of anti-B alloantibodies, plasma or WB from a type A cat would be preferred: type AB WB or plasma would be

ideal, but unrealistic in most settings given the low frequency at which type AB cats are found.

Hemolytic transfusion reactions in cats can be avoided by blood typing both the donor and recipient. Crossmatching is also important, especially if the intended recipient's transfusion history is unknown or if the recipient has been previously transfused. A crossmatch test (see Chapter 139) can identify preformed alloantibodies against a blood group antigen, thus preventing an AHTR. Until recently, blood types and naturally-occurring alloantibodies outside of the AB blood group system were not recognized, but incompatible crossmatches between AB blood type compatible cats have been described, suggesting the existence of other blood groups (see Chapter 92).^{20,44} *Mik*, a newly described blood group in cats, can also result in HTRs.⁴⁵ A cat negative for the *Mik*-RBC antigen can experience an AHTR, similar to those described in type A cats receiving type B blood, after transfusion with *Mik*-positive RBCs.⁴⁵ Given that this antibody was demonstrated to be naturally-occurring, crossmatching cats prior to even a first blood transfusion may be warranted. An incompatible crossmatch test, in the face of blood-type compatibility, suggests alloantibody formation and should not be ignored.

Some dogs may have naturally-occurring RBC alloantibodies, though there is controversy regarding their clinical significance. Generally, HTRs require prior sensitization through a previous blood transfusion or, to a much lesser extent, pregnancy.⁵ A DEA 1.1 negative dog who has been sensitized by transfusion of DEA 1.1 positive blood will develop anti-DEA 1.1 antibodies. A second transfusion with DEA 1.1 positive blood will cause an AHTR. Such a reaction has been documented clinically: a DEA 1.1 negative recipient had been sensitized to the DEA 1.1 antigen through a previous transfusion, and upon inadvertently receiving DEA 1.1 positive blood 3 years later exhibited a 2°C rise in temperature within one hour of transfusion and developed hemoglobinemia and hemoglobinuria.¹⁷ Following an AHTR plasma will be red-tinged due to hemoglobine-mia (Fig. 100.1); this is most often identified when a post-transfusion PCV is measured. An AHTR is not exclusive to the DEA 1.1 antigen; alloantibody production and HTRs have been documented for other RBC antigens in dogs, including known DEA, as well as RBC antigens without DEA designation.^{4,9,31} When a compatible donor is not easily identified on a blood crossmatch, testing both siblings of the patient or potentially dogs of the same breed has been shown to be successful in identifying a compatible donor.^{4,9}

Delayed Hemolytic Transfusion Reactions

Delayed hemolytic transfusion reactions (DHTRs) are defined as those occurring more than 24 hours following a transfusion, but the time of onset can vary to 48 hours post-transfusion depending on the reference.¹² DHTRs are often the result of an anamnestic response to a RBC antigen that the recipient lacks, with the previously produced reactive alloantibody weak or present

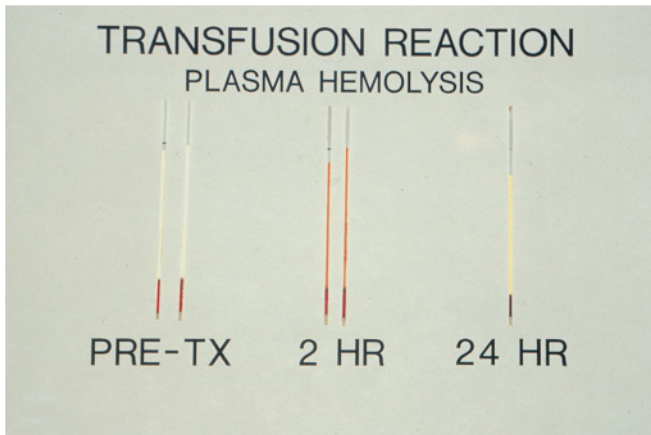


FIGURE 100.1 Serial packed cell volumes (PCVs) from a dog experiencing an acute hemolytic transfusion reaction. Note the transfusion of plasma from clear to hemolyzed to icteric over 24 hours. As a consequence of the transfusion reaction, the PCV fell following the transfusion. (Courtesy of Dr. Ann Hohenhaus.)

in very low concentrations. Pre-transfusion crossmatch testing may not identify the potential incompatibility if the test is insufficiently sensitive. Alternatively, DHTRs may be a result of primary alloimmunization to a RBC antigen, with hemolysis occurring weeks later after sufficient time for alloantibody production.¹²

A DHTR should be suspected if there is a more rapid decline than expected in PCV in the weeks following a blood transfusion, based on the patient's underlying disease. Additional clinicopathologic signs to support a diagnosis of DHTR include hyperbilirubinemia, bilirubinuria, or hemoglobinuria: fever can develop but often goes unnoticed. Diagnosis of a DHTR requires documentation of development of antibodies by the recipient against the donor(s) RBCs.

Preventing HTRs requires knowledge of blood groups present within a species, presence of naturally-occurring antibodies, prior transfusion history, and blood crossmatch testing. Regardless of the designation of acute versus delayed, the majority of HTRs can be prevented through proper blood typing and crossmatch testing.

Non-hemolytic Transfusion Reactions

Febrile and Allergic Reactions

Febrile and allergic reactions are two of the most common types of transfusion reactions reported in veterinary medicine, comprising 60–90% of reported reactions.^{20,24,25} Febrile, non-hemolytic transfusion reactions (FNHTRs) are defined in human medicine as a rise in temperature of either 2°F or 1°C during or within 4 hours post-transfusion without an obvious other cause for the rise in temperature.⁴⁶ Additional clinical signs can include vomiting and tremors: the vomiting will often resolve without treatment, but a decreased transfusion rate may help to alleviate this symptom.^{10,36}

Febrile, non-hemolytic transfusion reactions are frequently attributed to recipient alloantibodies which react with histocompatibility leukocyte antigens or other antigens present on donor lymphocytes, granulocytes, or platelets.^{6,36} Cytokines released from leukocytes and platelets within stored blood are also implicated as causes for FNHTRs and some allergic-type reactions.^{27,46} Pre-storage leukoreduction has been shown to decrease, but not eliminate, FNHTRs. Interestingly, leukoreduction of human platelet products did not significantly decrease the rate of allergic reactions, which were attributed to platelet-derived chemokines.^{6,27} Leukoreduction of canine WB does not decrease RBC viability but, for maximum reduction of leukocytes, needs to be performed on cooled blood following collection.⁷ Extrapolating from human data, leukoreduction at the time of administration would be less effective in veterinary medicine for decreasing FNHTRs; ideally, leukoreduction should be performed within a few hours of collection to minimize cytokine production and thereby prevent febrile reactions. However, routine leukoreduction of veterinary blood products is difficult to justify given the cost of the special leukoreduction filters and limited potential benefit to the majority of recipients. Platelet antigens would remain a potential source of reactivity even in leukoreduced WB or pRBCs. Recommendations for management of FNHTRs include stopping the transfusion but maintaining the intravenous line open with saline while excluding an AHTR or transfusion-related sepsis due to bacterial contamination of the unit (Fig. 100.2).

Allergic reactions are more often associated with transfusion of plasma products and are triggered by exposure to a substance, likely a protein, present in donor plasma to which the recipient has been sensitized.³⁶ Signs of an allergic reaction typically start within the first 15 minutes of a plasma transfusion but can occur during or within a few hours of administration. Clinical signs include mild to sometimes dramatic urticaria, pruritis, and erythema (Fig. 100.3). Additional signs can include vomiting, nausea, diarrhea, and/or abdominal pain.¹² Treatment of an allergic reaction to a plasma product includes stopping or, at least, decreasing the rate of transfusion, as well as administering an antihistamine such as diphenhydramine (1–2 mg/kg IM). Although pretreatment to prevent these types of immune reactions has been previously advised, administration of diphenhydramine to humans prior to leukoreduced platelet transfusions did not decrease the frequency of allergic-type urticarial reactions.⁴¹

Uncommon Immune Reactions

Post-transfusion purpura (PTP) is a relatively uncommon transfusion reaction in humans and has been described once in veterinary medicine.^{36,43} Thrombocytopenia develops 1–2 weeks post-transfusion due to recipient-produced anti-platelet antibodies. These antibodies are most often directed against a platelet specific antigen (e.g. in humans, HPA-1a) which the recipient lacks: platelets targeted for destruction are

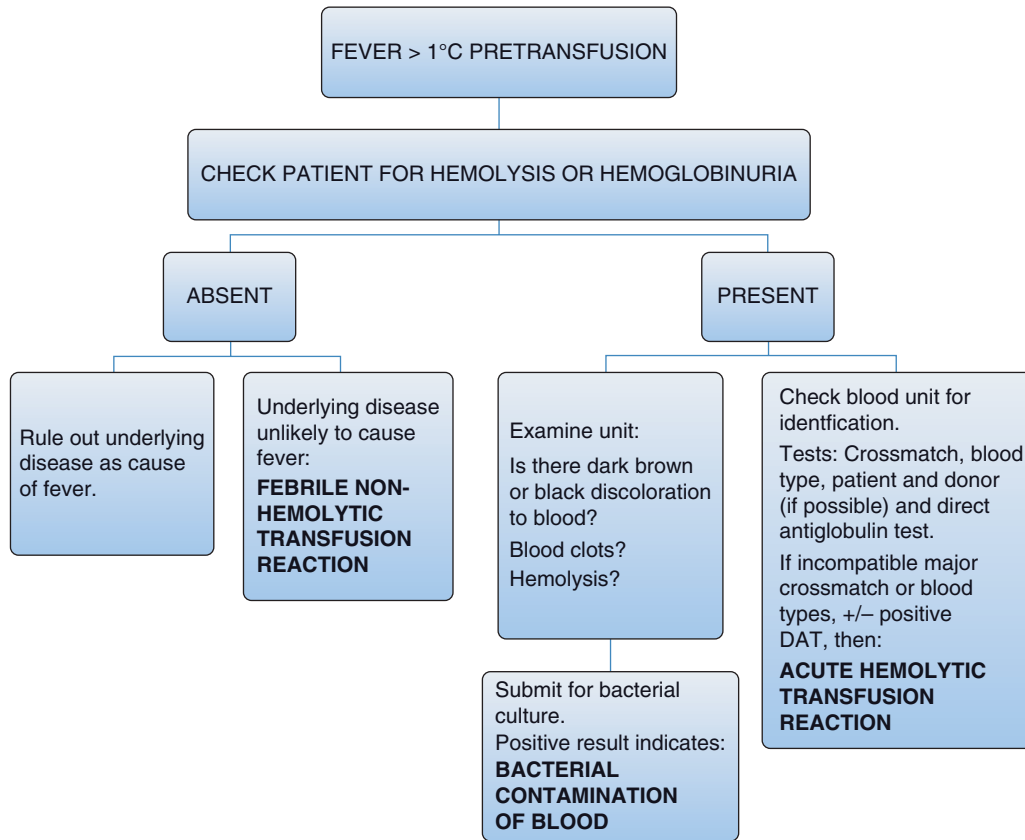


FIGURE 100.2 Algorithm for evaluation of a patient with a febrile transfusion reaction.



FIGURE 100.3 An urticarial reaction in a dog receiving plasma to treat rodenticide intoxication. Treatment with antihistamines and steroids induced resolution in 24 hours. (Courtesy of Dr. Ann Hohenhaus.)

typically those of the donor but targeting of recipient/patient platelets has also been documented.³⁶ PTP must be differentiated from worsening underlying disease or development of a concurrent disorder.

Anaphylaxis secondary to IgA deficiency has been reported in a single dog.² Anaphylaxis results from patient anti-IgA antibodies reacting to IgA antibodies in the donor plasma.² Clinical signs vary from facial

swelling, urticaria, and erythematous skin to respiratory distress, vomiting, diarrhea, and shock.¹ An IgA-deficient patient who previously demonstrated a severe anaphylactic reaction would require future transfusions that are free of IgA.

Transfusion-related acute lung injury (TRALI) is characterized by dyspnea, hypoxia, pulmonary edema, and fluffy, bilateral infiltrates on thoracic radiographs which develop during or within 6 hours of a transfusion. To date, TRALI has been described only in humans.^{35,39} Transfusion-related acute lung injury clinically mimics acute respiratory distress syndrome, circulatory overload, or cardiac failure, so it could be easily misdiagnosed. Fever may or may not be present.³⁹ Transfusion-related acute lung injury may result from several mechanisms, including both antibody-mediated pathways and non-immune causes, with neutrophil activation and subsequent leakage from capillaries (pulmonary microvasculature leakage) being the common final pathway.^{35,39} Treatment typically involves oxygen therapy and ventilation, when needed.³⁶

Transfusion-associated graft-versus-host-disease (TA-GVHD) also has yet to be reported in veterinary medicine. It results from the proliferation of viable donor T lymphocytes, which go unrecognized as foreign by the recipient's immune system; these donor T lymphocytes recognize and subsequently attack the recipient (or host).¹² In humans, certain cellular immunodeficiencies predispose the transfusion recipient to

this reaction; also, recipients of blood transfusions from close relatives place the recipient at greater risk for TA-GVHD.¹² Since leukoreduction is insufficient in preventing TA-GVHD, component irradiation is recommended in patient populations at increased risk of developing TA-GVHD (e.g. hematopoietic progenitor cell transplant recipients).¹² Clinical signs of TA-GVHD in man reportedly manifest within 2–50 days of transfusion and include a rash, diarrhea, liver dysfunction and pancytopenia. Mortality is greater than 90%.¹²

NON-IMMUNE COMPLICATIONS

Infectious Disease Transmission

Transmission of infectious agents to a blood transfusion recipient, though a relatively infrequent complication in both human and veterinary transfusion medicine, is not a true transfusion reaction but is obviously an avoidable and potentially devastating complication of transfusion. Infectious diseases documented to have been transmitted via transfusion in veterinary medicine include babesiosis, leishmaniasis, bartonellosis, and diseases caused by *Mycoplasma* sp.^{3,13,14,29,30,34,37,40} Although WB and pRBCs carry a greater risk of infectious disease transmission, plasma is not without risk; recently, herpesvirus was inadvertently transmitted to horses receiving commercially prepared plasma.³ A recent consensus statement from the American College of Veterinary Internal Medicine includes guidelines for recommended testing for both canine and feline blood donors based on the literature and known blood-borne agents (see Chapter 94).⁴² Documentation of a transfusion-acquired infection requires testing of pre-transfusion recipient blood, in addition to testing the donor; unfortunately, pre-transfusion recipient samples are not always available. Proper transfusion records will allow for easy identification of the suspected infected donor, removal of any remaining blood units from that donor, and appropriate treatment of the donor.

Transfusion-associated Sepsis

Bacterial contamination of a blood unit can result in transfusion-associated sepsis. Clinical signs associated with transfusion of a contaminated blood unit include fever, vomiting, diarrhea, hypotension, and hemolysis.¹² Contamination can occur at the time of collection from inadequate preparation of the venipuncture site or contamination of materials used in collection. Once contamination of a unit occurs, bacterial numbers tend to increase with storage time, even when the unit is refrigerated. Storage at room temperature, which is necessary to preserve the post-transfusion survival of platelets in fresh WB and platelet-rich preparations, can also lead to bacterial proliferation in contaminated units. It has been documented that units of human pRBCs stored for longer than 21 days are more likely to contain proliferating bacteria.¹⁸ The severity of a reaction will ultimately depend on the bacterial species, the number of bacteria

present, and the clinical condition of the recipient/patient.²¹

Reports of bacterial contamination of blood products and transfusion-associated sepsis are infrequent in veterinary medicine.^{2,22} Contamination of feline blood units with *Serratia marcescens* was attributed to contaminated supplies used in donor collection. Vomiting was the most common clinical sign seen in cats receiving contaminated units, and four recipients died.²² This report illustrates the importance of bacterial culture of materials other than the unit and demonstrates the steps necessary to identify the source of contamination. Contaminated WB or pRBC units are typically discolored (dark brown, purple, or black), and clots and air bubbles may also be present.^{22,36} The discoloration associated with bacterial contamination indicates deoxygenation, hemolysis, and the formation of methemoglobin. Blood units with such an abnormal appearance should not be used, and an investigation to confirm bacterial contamination and identify the source of contamination should be pursued. Lastly, administration of a blood component unit should ideally take no longer than 4 hours to reduce the potential for bacterial proliferation. Once a unit is opened, even if refrigerated, it should be used within 24 hours. If bacterial contamination is suspected after the transfusion has been started, the transfusion should be stopped immediately and the unit submitted for a Gram stain and bacterial culture. Blood culture of the recipient may also be warranted.

Uncommon Non-immune Complications

Citrate Toxicity

Citrate is the anticoagulant used in most standard blood collection systems. Plasma and WB have the greatest concentrations of citrate when compared to pRBCs. Patients most at risk for citrate toxicity include those receiving large volumes of blood products, such as in cases of massive transfusion (see Chapter 95) and, potentially, patients with severe liver disease since citrate is metabolized by the liver. Ionized hypocalcemia and hypomagnesemia result from chelation of these cations by citrate, and associated clinical signs include muscle tremors, vomiting, cardiac arrhythmias, ear twitching, hypotension and/or tetanic seizures.^{2,24} Intravenous calcium can be administered to treat clinically significant transfusion-related hypocalcemia.

Circulatory Overload

Patients with chronic anemia and a compensatory expanded plasma volume or patients with compromised pulmonary and/or cardiac function may be at risk for circulatory overload.³⁶ Expected clinical signs include respiratory distress and, in some cases, congestive heart failure; signs may be seen during or soon after transfusion. Management of fluid overload with diuretics is advised. Slowing the transfusion rate and using pRBCs rather than WB in anemic patients can help to decrease the risk of circulatory overload.

Non-immune Hemolysis Unrelated to Bacterial Contamination

Hemolysis unrelated to a blood group incompatibility or bacterial contamination may occur in vitro due to improper storage or handling of blood units, including use of a hot waterbath, inadvertent freezing, use of pumps not approved for administration of blood products, and concurrent administration of incompatible fluids, such as 5% dextrose or hypertonic saline through a shared intravenous line.^{32,36,38} Warmed blood has been demonstrated to have decreased survival in the patient which could mimic a delayed transfusion reaction.³²

PREVENTION AND RECOGNITION OF TRANSFUSION REACTIONS

Prior to blood product administration, baseline values of temperature, heart rate, and respiratory rate should be determined and pre-transfusion PCV should be recorded (see Chapters 95–99). Pre-transfusion WB and serum samples should be collected from the recipient, if not already submitted for other routine testing. These samples can be invaluable in cases of HTRs and in documentation of infectious disease transmission. Transfusion of any blood product is ideally started at a slower rate for the first 15 minutes while monitoring for signs of any adverse reactions. Clearly, in cases of severe, ongoing blood loss, rapid administration from the start may be necessary. Transfusion monitoring should be performed every 15 minutes for the first hour, with serial measurement of temperature, heart and respiratory rates. If there is any concern about an AHTR, evaluation of plasma and/or urine for the presence of hemoglobin is indicated. Lack of an expected increase in PCV post-transfusion may also be suggestive of an AHTR.

EVALUATION OF A PATIENT WITH A SUSPECTED TRANSFUSION REACTION

Investigation of hemolytic and febrile reactions should include inspection of the patient's plasma for evidence of hemolysis or icterus; evaluation of the blood product unit administered including unit labels, the administration set, Gram stain and blood culture of the transfused product; comparison of patient bilirubin both pre- and post-transfusion; retesting of patient and donor blood type, especially for reactions involving RBC-containing units; and post-transfusion crossmatch testing of recipient-donor compatibility. If a crossmatch test was performed pre-transfusion, it should be repeated in the case of a HTR; if one was not performed, a crossmatch test should be done using both pre- and post-transfusion plasma/serum, if available. In general, if a transfusion reaction is suspected, the transfusion should be stopped but the intravenous line kept open with saline until the patient and blood product can be further evaluated.

Adequately screening potential blood donors, transfusing patients only when clinically indicated, selecting the most appropriate blood component, properly administering blood products, and carefully monitoring transfusion recipients will greatly diminish the risk of a transfusion reaction.

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Major Histocompatibility Complex Antigens

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Typing Paradigms
Serologic Typing

Cellular Typing
Molecular Typing
Species Variations

Acronyms and Abbreviations

CYP21, 21-hydroxylase; DSCP, double-strand conformation polymorphism; HSP 70, heat shock protein 70; LA, lymphocyte antigen; MHC, major histocompatibility complex; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; SSCP, single-strand conformation polymorphism; SSOP, sequence specific oligonucleotide probes; TNF, tumor necrosis factor.

The major histocompatibility complex (MHC), a large region of associated genes, is the backbone of the immune system for most animals, birds, reptiles, and amphibians.¹¹ Genes in this region are responsible for encoding proteins that control all aspects of antigen presentation and processing leading to disease resistance or immune tolerance. This region seems extremely polymorphic and highly conserved as a unit in most species evaluated to date.⁶⁷ Classical MHC is made up of three classes: I, II, and III. Class I MHC genes encode for surface glycoproteins or histocompatibility antigens found on all somatic cell surfaces. Responsible for presentation of endogenously derived peptides to the T-lymphocyte cell receptor, class I molecules mediate the destruction of virus and tumor through activation of CD8-positive, CD4-negative cytotoxic T cells and natural killer cells. Class II MHC genes encode for surface glycoproteins found on antigen-processing cells like B lymphocytes, dendritic cells, macrophages, and epithelial cells. Class II molecules present exogenously derived peptides and mediate the activation of CD4-positive, CD8-negative T-helper cells, resulting in the secretion of specific cytokines leading to cell mediated and humoral immune responses. Within the class II region of most MHC complexes lie genes that encode proteins responsible for endogenous antigen processing. For example, genes encoding subunits for the 20S proteasome (LMP2 and LMP7) are here. Proteasomes are responsible for intracellular peptide degradation.⁴⁵ These genes are inducible. Peptide transporter molecules (TAP1 and TAP2) move peptides derived by the 20S proteasome across the membrane of

the endoplasmic reticulum for loading into class I molecules for presentation. TAP genes are also within the class II region. It is interesting that genes encoding antigen-processing and genes presenting antigen to the immune system are linked in most species. The class III region of the MHC encodes for several complement factors (Bf, C2, C4), 21-hydroxylase (CYP21), tumor necrosis factor alpha and beta (TNF- α and - β), and heat shock protein 70 (HSP 70). Nomenclature for the MHC has included different names for different species with the lymphocyte antigen (LA) designation. Attempts at standardization have led to the suggestion of a system reliant on the first letters of the genus and species. For example, dog LA (DLA) would be replaced with *MHCCafa*.³⁴ Several species would retain their old designation: H-2/mouse, HLA/human, and RT-1/rat. This system is frequently seen in the literature but has not been accepted by the entire scientific community; therefore, both nomenclatures are in use.

TYPING PARADIGMS

Serologic Typing

Typing paradigms to recognize and define the highly polymorphic MHC genes and gene products (antigen expressed on the cell surface) have undergone evolutions. Complement-mediated cell lysis with absorbed complement and sera derived from multiparous or immunized animals or man was one of the early, serologic methods used.^{7,39} This assay is often referred to as

the microlymphocytotoxicity assay. It was run in Terasaki microtiter plates and used microliter quantities of reagents. The sera used were often validated through international workshops under the auspices of the International Society of Animal Genetics. Examples of sera validation are cited under species-specific information later in this chapter.^{8,12-14,32,35} Serologic typing can be used for both class I and class II antigen characterization.^{7,39}

Cellular Typing

Cellular typing was first used to recognize class I antigen and is still useful for determining functionality for the plethora of class II antigens seen in most species.¹⁵ Cellular assays include the use of the mixed lymphocyte culture or reaction.⁴³ In brief, lymphocytes of two test subjects are mixed together in culture. The lymphocytes of one subject are treated with either chemicals or radiant energy to render them incapable of response and serve as stimulator for the other population of cells. If stimulated, the untreated cells undergo lymphoblastogenesis. The intensity of the response is proportional to the increase in the number of cells. A second form of cellular typing is termed primed lymphocyte testing.⁴⁴ The format of this testing is very similar to a mixed lymphocyte culture, with treated and untreated cells used, except it often employs well-characterized cell lines that are used as a reference base.

Molecular Typing

Molecular methods now dominate the MHC typing modalities.⁹ Initially, these methods offered very crude estimates of alleles present by the performance of restriction fragment length polymorphism (RFLP) testing, often using xenogeneic probes. For many species this method represents the only testing data available. Further refinement in testing modalities has brought forward methods based on amplification of a portion of a MHC gene (usually exon 2 for class II and exon 3 for class I). The polymerase chain reaction (PCR) is most often used for this amplification.⁵⁴ Amplified fragments may be bound to a membrane and probed with sequence specific oligonucleotide probes (SSOP) that recognize the polymorphic position within the gene; this method is termed PCR-SSOP typing.^{9,66} Fragments may also be digested with endonuclease and the resulting RFLP patterns used to recognize polymorphic variation within the region tested; this is termed PCR RFLP.^{9,27} Secondary structure differences introduced by the sequence variation can be seen when the molecules migrate within a gel. Such structures can be single stranded or double stranded. Amplified fragments can also display secondary structure, and this characteristic is used to recognize variation seen in different MHC alleles. This type of testing, based on secondary structure, is termed single-strand conformation polymorphism (SSCP) typing or double-strand conformation polymorphism (DSCP) typing. When amplified products are used, the terms PCR SSCP or PCR DSCP are used.^{6,9}

Specific alleles can also be amplified with PCR in a testing protocol that allows for high-resolution typing.⁹ Briefly, primer pairs are anchored in the polymorphic regions of specific alleles. This methodology may share one of the primers with other alleles but each allele has a unique set. This type of testing is often referred to a PCR SSP.⁴⁹

The most definitive type of MHC typing is sequenced-based typing.⁹ This form of analysis allows for discrete allele assignments even in the presence of heterozygous alleles. One note of caution: although the detection of alleles can be accomplished at the molecular level, there is no assurance that these molecules may be expressed.⁵² This can lead to errors in expected outcomes of research protocols and transplantation results.

As a result of the numerous genomic mapping projects completed in many species, the identification of microsatellite markers within the MHC complex provides unique tools for haplotype studies within a family or related population.¹⁸ Microsatellite markers are short tandem repeats present in the genome. Primers flanking the tandem repeat are designed so that, when amplified, the product length variation depends on the number of repeats present. Because of the high degree of linkage disequilibrium or lack of recombination, the haplotype (full complement of MHC of one parental chromosome) can be followed throughout a family for pedigree, transplant, or disease association. Microsatellites may be used to track MHC alleles within a family but not outside the family or related species.¹⁶

Recent advances in molecular typing described above have allowed definition of the MHC in nonhuman species without the need for access to large serum antibody databases. Knowledge of MHC "type" allows several clinical applications: paternity testing, disease resistance prediction, and transplantation pairing. Paternity testing has been used in man and animals for the purpose of defining shared alleles. Because of the high degree of polymorphism within the MHC, unique inheritance patterns can be used to predict the likelihood of unknown paternity. Studies of the MHC in man and mouse have led to the association of MHC type with disease resistance, disease inheritance, and potential autoimmune disease. As a point of example, ankylosing spondylitis occurs in 96% of Caucasians demonstrating a class I haplotype, HLA-B27. This association of ankylosing spondylitis is also seen in rats, mice and primates carrying a B27-like haplotype.⁷¹ Frequent association of MHC haplotype and virally mediated leukemia has been made in several species, including the mouse, rat and bovine.⁶⁷

Transplantation in humans relied on MHC typing for selection of donor-recipient pairs for over 30 years.³⁶ Strong clinical evidence supports the theory that concise allelic matching minimizes the graft versus host disease seen in solid organ and bone marrow transplantation. With the advent of molecular typing techniques, nonhuman species may be evaluated for MHC type before transplantation in an attempt to maximize post graft survival. The algorithm provided (Fig. 101.1) is designed to offer a general means of selecting donor-recipient

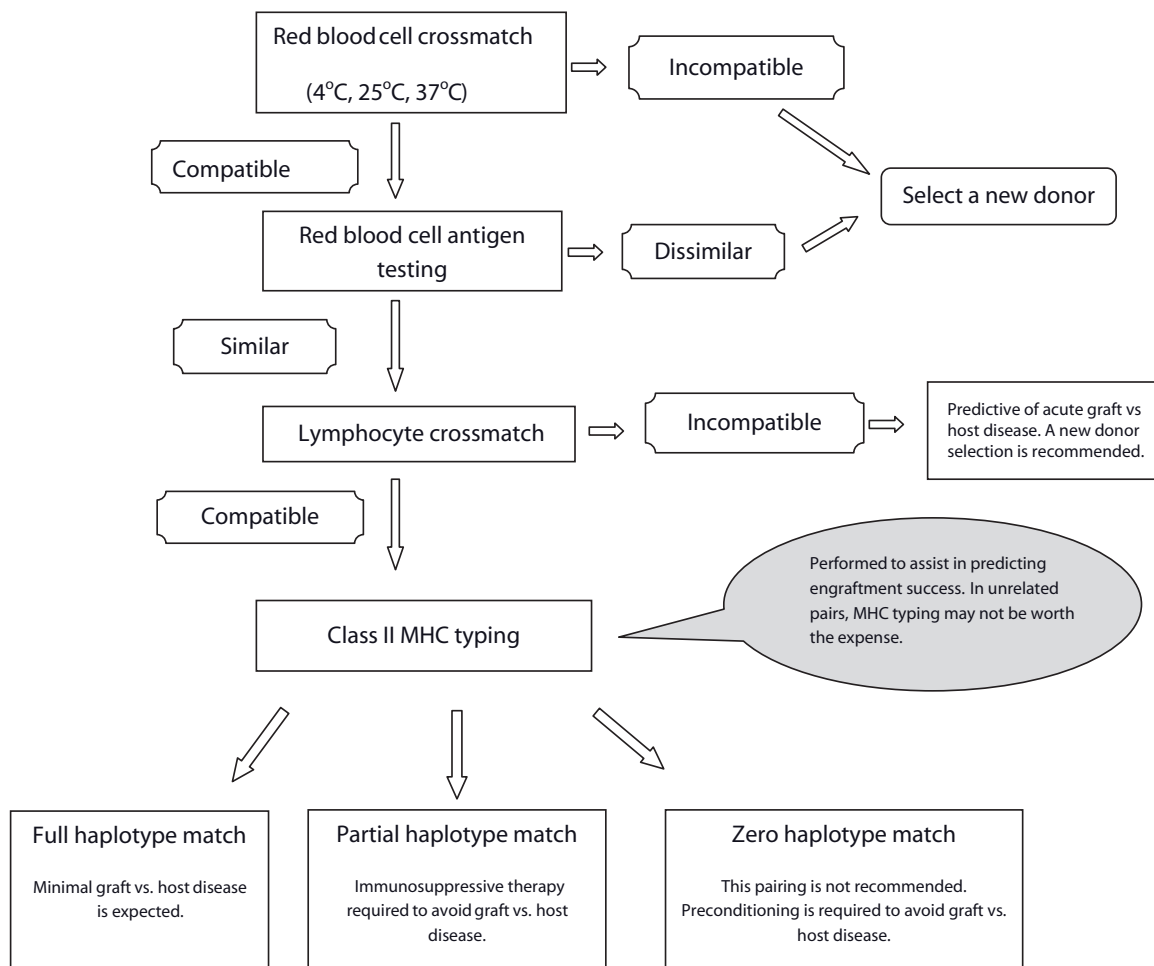


FIGURE 101.1 Donor-recipient pair selection for solid organ and bone marrow transplantation.

pairs for transplant in which MHC haplotype is used.²⁵ Recently, in nonhuman species, the use of protective conditioning through hematopoietic cell transplant and chimeric development has been explored to minimize immunosuppressive therapy post engraftment and expand the potential donor pool in highly polymorphic populations with limited donor organs.⁶⁵ Using this technique, the recipient is prepared through immunosuppression to receive peripheral blood stem cells from the donor. After engraftment is confirmed, the recipient receives the solid organ transplant. Early studies by Storb and others suggest that protective conditioning may offer a reasonable long-term outcome in the dog.^{21,22,65}

SPECIES VARIATIONS

The human MHC, often referred to as the HLA complex, is perhaps one of the most well-defined systems (Table 101.1). This characterization was initially performed by serologic and cellular techniques, and over the past 20

years by molecular methods.⁹ Allelic specific typing paradigms for all three classes of HLA genes have been developed with the progression of molecular science. HLA-A, HLA-B and HLA-C are traditional class I genes present on most nucleated cells of man; non-traditional genes, HLA-E,-F,-G,-H,-J,-K and -L are also present.⁶⁷ The class II regions have HLA-DR, HLA-DQ, and HLA-DP genes. The numbers of DRB genes present can vary, depending on which DRB gene is present. Within the class II region are the antigen-processing and transporter molecules, TAP and LMP. The class III region contains genes for HSPs and the CYP21 responsible for steroid metabolism. TNF genes are also present in the HLA class III region; from an evolutionary perspective, genes in this region are thought to be precursors to class I and class II genes. There are numerous microsatellites in the HLA region suitable for haplotype assignment and disease association studies.¹⁸

Primate MHC is very similar to that of man.⁷¹ Class I products are defined as -A and -B. Higher monkeys such as the chimpanzee also have a -C locus. The class II molecules vary by Old World or New World species.

TABLE 101.1 Characterization of Animal MHC Systems

Species	Nomenclature	Class I	Class II	Class III	Unique Feature
Human	HLA	A, B, C	DR, DQ, DP	C2, C4, TNF, HSP, CYP21	
Rhesus	RhLA (Mamu)	A, B			
Chimpanzee	ChLA (Patr)	A, B, C	DR, DQ, DP	C2, C4, TNF, HSP, CYP21	
Mouse	H-2	K, D, L	A, E	Bf, C2, C4, M, TNF, HSP70	
Rat	RT1	A	DR, DP	Bf, C2, C4, TNF, HSP70	
Guinea Pig	GPLA (Capo)	B	DR	Bf, C2, C4, TNF, HSP70	No DP
Rabbit	RLA (Orcu)	A	DR, DQ, DP	Not linked	
Canine	DLA (Cafa)	88, 12, 64, 79	DR, DQ, DP	C2, C4, TNF, HSP, CYP21	
Feline	FLA (Feca)	H, K, E	DR, DQ	C2, C4, TNF, HSP70	
Bovine	BoLA (Bota)	A, B	DR, DQ, DY	Bf, CR, CYP21, HSP70, M	No DP
Ovine	OLA (Ovar)	N	DR	C4, CYP21, TNF	
Caprine	CLA (Cahi)	N	DR, DQ	C2, C4, TNF, HSP, CYP21	
Equine	ELA (Eqca)	A	DR, DQ	C4	
Swine	SLA (Sudo)	1, 2, 3, 6	DR, DQ	Bf, C2, C4, TNF, HSP70	Class II on resting T cells
Chicken	B complex	F	L	Not linked	Expression on RBC
Reptilian	Species specific	U	D	Data not available	
Amphibian	Species specific	A	DR	Data not available	
Fish	Species specific	U, Z/Ze	D	Data not available	

Old World monkeys demonstrate -DR, -DQ, and -DQ class II genes;⁵³ New World monkeys have dropped some of these class II alleles.⁶² Class III genes include Bf, C2, C4, CYP21 and HSP70.¹ Microsatellites for many primate species exist, like the microsatellite facilitating rapid DRB haplotyping in Rhesus macaques.^{16,62}

The mouse MHC, H-2, has been extensively characterized by serologic, cellular, and molecular techniques.³³ Situated on chromosome 17, the H-2 is organized in a similar fashion to the human MHC. Mouse class I equivalents include H2-K, -D and -L. Class II is made up of H2-A and -E. Class III is found exactly patterning the human MHC.⁶⁷ Many microsatellite markers have been characterized within the H-2. Strains of mice have been developed demonstrating H-2 disease associations; examples include leukemia and Gross virus infection.

The rat MHC, R-1, is homologous with the human MHC to a large degree. However, the genomic sequence of the rat MHC has recently been published indicating a significant difference in class I molecules when compared to the human MHC. Class II and III molecules maintain homology with the human MHC.²⁶ A model of multiple sclerosis, myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalitis, can be characterized as acute versus remitting/relapsing based on RT1-A, a class I molecule. This association reinforces the strong role that MHC plays in disease resistance and susceptibility.

The guinea pig (*Cavia porcellus*) MHC has class I, II, and III gene products.⁷² Class I products have been identified by serology and molecular methods; GPLA-B is the only class I molecule currently defined. Class II molecules include a -DR homologue, the -I region.^{59,64} Class III gene products include Bf, C2, C4, TNF and HSP70.¹⁹

The rabbit MHC, or rabbit LA (RLA), has been largely characterized through molecular techniques. RLA-A is a class I molecule; RLA-DR, RLA-DQ, and RLA-DP are class II gene products. Class III is not linked physically to the class I and class II genes as in most other species.⁷³

The canine MHC (DLA) complex was initially characterized by both serologic and cellular assays.^{55,56} Both class I and II antigens are present.^{8,14,32} Class I genes include DLA-88, DLA-12, DLA-64 and DLA-79. DLA-88 is highly polymorphic.^{31,68-70} With the advent of molecular typing, the class II genes have been well defined.³² The DLA-D region of the dog has -DR, -DQ, and -DP homologues as seen in man, as well as -DO and -DN homologues and an LMP-2 gene. The class III region in the dog has C2, C4, CYP21, and TNF genes present.⁵⁷ Numerous microsatellites have been described for haplotype description in purebred and mixed breed dogs.^{10,32,68} Despite frequent use of the dog as a transplant model for human preclinical studies, easily accessible molecular typing of DLA is not available.²¹

The feline MHC (FLA) could not be characterized until the advent of molecular typing. Cats do not readily form the anti-FLA antibody necessary for routine serologic identification. Until molecular characterizations were performed, the cat was considered uniquely monomorphic.^{74,75} Class I, class II and class III equivalents have been identified on chromosomes B2 and 3. Three class I genes (FLA-H, FLA-K, and FLA-E) are described.⁷⁴ Class II genes have also been described (FLA-DR, FLA-DQ). The importance of FLA-DR in skin allograft suggests that MHC typing and matching prior to transplant may extend graft longevity.

The bovine MHC, bovine LA (BoLA), has been well characterized by both serological and molecular methods.^{12,13} Cattle have BoLA-A and -B class I homologues. BoLA-DR and -DQ are class II molecules. Cattle

do not have a -DP homologue but do have a third class II gene expressed, BoLA-DY. The BoLA system has been associated with the susceptibility and resistance to several pathogens.⁶⁷ Cattle have the following class III genes linked to the BoLA complex: Bf, C4, CYP21, HSP70, and the blood group M.³⁷ Recent evaluations of BoLA-DQA indicate susceptibility to mastitis based on haplotype.

The ovine MHC, ovine LA (OLA) complex, has been primarily defined by molecular methods.⁴² There are OLA-A and OLA-B class I genes. Class II genes include OLA-DR, -DQ, -DN, -DM and -DY.⁷³ Class III genes are described, together with multiple microsatellite markers.⁶⁰

The MHC complex of goats, caprine LA (CLA), is less well defined than those of other ruminants.⁴⁷ Molecular sequencing has allowed the definition of the class I molecule, as well as a -DR region representing a class II molecule.^{1,2,76} Class III molecules are homologous with the human MHC.

Equine LA (ELA) has been characterized by serologic, cellular and molecular methods.^{35,38} The loci defined are ELA-A, ELA-B, and ELA-C for class I, and ELA-DR and -DQ for class II molecules. Class III molecules are present but have not been fully defined.^{23,24}

The swine MHC, swine LA (SLA), has been characterized by serologic, cellular and molecular techniques.⁵⁸ This gene cluster has been found to be the smallest MHC in mammals tested to date. Class I, class II and class III molecules are present. SLA-1, -2, and -3 are class I molecules.³ Class II molecules include SLA-DR and SLA-DRB. Several microsatellite markers on chromosome 7 within the SLA gene complex are available.^{4,63} Recent interest in the use of the miniature swine as a research model for xenotransplant has spurred the completion of MHC mapping the pig.

The chicken MHC, B complex, is unique in that it has class I, II, III, and IV gene products present. Class I genes are defined as F, whereas class II genes are L. Unlike other MHC complexes reviewed, chicken class III products are not physically linked to the class I or II genetic regions. Class IV gene products (-B,-G), unique to the chicken and reptile, are expressed on red blood cells.²⁹

Reptilian MHC is now predominantly defined by molecular methods. Class I and class II molecules with homology to the human MHC have been described.^{17,40,41} Definition of non-avian reptilian MHC is ongoing through sequence analysis, but limited by sample availability.

The amphibian MHC has received much attention in the past 3 years as scientists search for reasons for species variation and decline.^{5,61} Molecular methods have allowed an economically feasible means of determining the presence and defining the sequence of major histocompatibility molecules.³⁰ Class I and class II molecules, as well as a loosely linked class III molecular region, have been identified in several species of amphibians.²⁸ Unique to amphibians, the class I molecule is extremely stable with only one class I gene found

in species separated for up to 100 million years. A great deal of work has been performed on *Xenopus*, a species of clawed frog.⁴⁶

Evaluation of the MHC in fish has largely been connected with the advance of aquaculture.⁵¹ Similarly to the poultry industry, aquaculturists have found the MHC to be useful in predicting disease resistance and parasite vulnerability.⁵⁰ Molecular methods have allowed a detailed body of work to develop defining class I (-U, -Z/ZE) and class II (-D) molecules in fish.^{20,48}

Classical MHC genes have been defined more completely by molecular methods in the past decade. The importance of this gene region to adaptive immune response, disease resistance, and population genetics has been enhanced as our testing methods have advanced. Through evaluation of this gene complex, clues to the evolutionary history and future of all species can be found.

In 2003, the Immuno Polymorphism Database⁷³ was developed. Established by the HLA Informatics Group of the Anthony Nolan Research Institute, this free database offers a section solely dedicated to sequencing data relating to the MHC (IPD-MHC) and provides a concise way for scientists to gather, share, and evaluate the MHC of vertebrate species.

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Hematopoietic Stem Cell Transplantation

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Types of Transplantations

Donor Types

Allogeneic transplantation

Syngeneic/Autologous transplantation

Xenogeneic transplantation

Bone Marrow

Whole marrow

Purified hematopoietic stem cells

Mesenchymal stromal cells

Hematopoietic Stem Cell Purification

Stem Cell Markers

Sources

Bone marrow

Peripheral blood

Umbilical cord blood

Transplantation Methodology

Donor Selection

Recipient Conditioning

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Outcomes

Complications of Conditioning and Transplantation

Current and Future Applications

Current Treatment Applications

Future Potential: "Transdifferentiation?"

Acronyms and Abbreviations

BMT, bone marrow transplantation; DLA, dog leukocyte antigen; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; GVHD, graft versus host disease; GVL, graft versus leukemia; HVG, host versus graft; HLA, human leukocyte antigen; HSC, hematopoietic stem cell; HSCT, hematopoietic stem cell transplantation; LA, leukocyte antigen; MAPC, multipotent adult progenitor cell; MHC, major histocompatibility complex; MPC, myeloid progenitor cell; MSC, mesenchymal stromal cell; PBMC, peripheral blood mononuclear cells; TBI, total body irradiation; UCB, umbilical cord blood.

*"... all the wealth I had ran in my veins."
William Shakespeare, The Merchant of Venice*

The concept of a pluripotent stem cell capable of recapitulating all the cell lines of the hemolymphoid system was born from experiments with lethally irradiated mice in the early 1960s. However, it was not until 1988 that hematopoietic stem cells (HSCs) were prospectively isolated from mice.⁶¹ The prevailing model of hematopoiesis is hierarchical and holds that mature, differentiated cells arise from primitive HSCs (see Chapter 3). Recently, a new model of HSC regulation has been put forth that represents a transformational view of hematopoiesis.^{32,48} This view is of a continuous rather than hierarchical model of HSC regulation, and postulates that HSCs vary in phenotype according to cell cycle stage.⁴⁶ Cell cycle-dependent traits include *in vivo* engraftment, progenitor numbers, expression of adhesion proteins and cytokine receptors, and differentiation

into megakaryocytes and granulocytes.⁴⁸ Clearly, if shown to be accurate, a continuum model of HSC regulation will impact upon current transplantation strategies, as well as the concept of a rigid HSC phenotype.

Bone marrow provided the source of HSCs in the original transplantations in the late 1950s. The first bone marrow transplantations (BMTs) in humans were performed with little to no knowledge of transplant antigens or engraftment mechanisms, and results were less than promising. It was the use of animals in research, especially dogs, which helped realize the clinical potential of BMT in humans. The discovery of specific HSC markers opened the door to non-marrow sources of HSCs and expanded our knowledge of HSC biology. Stem cell biology both derives from and contributes to transplantation science. We continue to rely upon animals to further our knowledge of stem cell biology and translate therapeutic strategies into clinical practice.

TYPES OF TRANSPLANTATIONS

Donor Types

Hematopoietic stem cell transplantations (HSCTs) can be syngeneic, allogeneic, or xenogeneic. These donor types relate to the genetic and, therefore, antigenic relation between donor and recipient. Syngeneic transplants are derived from monozygotic twins, animals of the same genotype (i.e. mice of the same strain), or autologous HSCTs. In allogeneic transplants, HSCs are intraspecific and the genotype differs from the recipient, whereas xenogeneic transplants are between species. Table 102.1 provides a comparison between the three types of transplantation donors.

Allogeneic Transplantation

Allogeneic transplantations are further subcategorized according to both histocompatibility and to their relation to the recipient. Immunosuppressive therapy is required for allogeneic HSCT in order to suppress both host and donor responses; the former is necessary for engraftment, whereas the latter prevents graft versus host disease (GVHD). GVHD is one of the most significant impediments to allogeneic HSCT and results from immune targeting of host cells by the donor T cells. For many years the GVHD associated with allogeneic HSCT precluded its clinical application, but advances in immune modulation, donor and HSC selection, and supportive therapy have greatly decreased many of the adverse effects. However, GVHD can be exploited to eliminate host leukemic cells; allogeneic HSCT is more effective than autologous HSCT in tumor killing. This phenomenon is referred to as “graft versus leukemia” (GVL). The GVH effect may also eliminate host autoreactive T cells; thus HSCT is now being explored for the treatment of autoimmune diseases.⁶⁵ For diseases in which the recipient’s HSCs are inherently defective (e.g.

severe combined immunodeficiency, Fanconi anemia), allogeneic transplantation is currently the best option.

Syngeneic/Autologous Transplantation

With autologous or syngeneic transplantation, there is no genetic incompatibility, which obviates the need for post-transplantation immune suppression, the risk of GVHD, and engraftment failure. On the other hand, autologous HSCT does not provide the benefits of GVL. Autologous HSCT is principally used as a rescue therapy following myeloablative therapy for hematologic malignancies. Interestingly, autologous HSCT does appear to have some success in the treatment of some autoimmune disorders. The positive effect is correlated with early disease stages and very intensive conditioning regimens.⁶⁵ The mechanism by which this occurs is thought to be a “re-setting” of the immune system following ablation of the existing hematology-lymphoid system; HSCT recapitulates ontogeny with concomitant re-self-tolerization of T cells.⁶⁸

As gene therapy advances, applications for autologous HSCT will expand when the patient’s own HSCs can be corrected *ex vivo* and returned, permitting autologous HSCT therapy for genetic conditions. Select animal models of genetic disease have been successfully treated by *ex vivo* gene therapy and HSCT.⁴

Xenogeneic Transplantation

One of the biggest obstacles to the use of xenogeneic HSCs is graft rejection. While graft rejection remains a significant hurdle to overcome, a benefit of xenogeneic HSCT is the near absence of GVHD.¹⁸ To date, xenogeneic HSCTs are used mainly for research rather than therapy; in a notable exception for an AIDS patient, the HSCT proved unsuccessful.⁴⁰ Several rationales are presented for therapeutic xenogeneic transplantation. The

TABLE 102.1 HSCT Donor Types: Indications and Consequences

	Allogeneic ^a	Autologous	Xenogeneic
Indications	Leukemias Lymphoma Genetic diseases Autoimmune diseases Myelodysplasia	Marrow rescue Gene therapy for genetic disease Autoimmune diseases	Tolerance induction for organ xenografts Genetic diseases Infectious diseases (e.g. AIDS)
Advantages	GVL Gene correction	No immune suppression Donor availability No GVHD No transmission of infectious agents	Donor availability Gene correction No GVHD
Disadvantages	GVHD Immune suppression Donor availability Graft rejection Donor transmission of infectious agent	No GVL	Xeno- or zoonoses Immune suppression Graft rejection Ethics (animal rights)

^aGVL, graft versus leukemia; GVHD, graft versus host disease.

first is that xenogeneic transplants offer a readily available supply of donors. Secondly, for infectious diseases such as AIDS, HSCT from a resistant species would reconstitute the hemolymphoid system with resistant cells; the flipside of this argument is the potential for zoonotic infection or, worse, introduction of a new pathogen to humans (xenozoonosis). Finally, xenogeneic HSCT can induce tolerance for solid organ xenografts.²⁵

Bone Marrow

Whole Marrow

Historically, marrow was used to reconstitute marrow. In addition to HSCs, marrow also contains other cells that have been shown to affect engraftment, GVHD, and tumor immunity; i.e. T cells, mesenchymal stromal cells (MSCs), and myeloid progenitor cells (MPCs). Marrow grafts can now be modified by decreasing proportions of the deleterious components responsible for GVHD while enriching for MSCs, MPCs, and CD34+ cells that can enhance engraftment and reconstitution.⁸ T cell modulation for HSCT is an ongoing challenge. Whereas T cell depleted marrow will produce little to no GVHD, graft rejection is very high due to the role donor T cells play in promoting engraftment.⁴⁴ There is also significant loss of GVL effects with T cell depleted marrow.

Purified Hematopoietic Stem Cells

The rationale for the use of purified or enriched populations of HSCs rests in improving engraftment and preventing GVHD. The poor engraftment following depletion of donor T cells to prevent GVHD can be reversed by increasing the dosage of CD34+ cells.⁴⁹ Recent studies have shown that HSCTs enriched for CD34+ cells can overcome even haploidentical (genetically related but MHC-mismatched) transplantation barriers by inducing tolerance in residual host T cells. The mechanism of tolerance involves tumor necrosis factor- α mediated deletion of host T cells by a CD34+ cell subset.⁵⁰

However, the use of pure CD34+ cell populations for HSCT has two big disadvantages: delayed immune reconstitution (as a result of the lack of donor T cells and conditioning-induced host T cell depletion) and high leukemia relapse rates (due to the lack of GVL). Delayed immune reconstitution puts the host at increased risk for serious infections. In veterinary medicine, the ability to purify HSCs is limited to those species for which anti-CD34 antibody is available, obviating the use of purified HSCs for all other species. The expense and technical expertise necessary for CD34+ cell isolation and culture further limit its practical use for most veterinary patients.

Mesenchymal Stromal Cells (MSCs)

Bone marrow contains at least two types of stem cells: HSCs and MSCs. The prevalence of MSCs in marrow is

10–100 times less than that of HSCs. MSCs have been described in rats, mice, humans, and dogs, and can be isolated from marrow and peripheral blood.^{29,31,45} MSCs are plastic-adherent when maintained in standard culture conditions; CD105+, CD73+ and CD90+; CD45– and CD34–; and able to differentiate *in vitro* into osteoblasts, adipocytes, and chondroblasts.¹⁷ In HSCT clinical trials, MSCs were non-immunogenic and exerted immunomodulatory effects that promoted engraftment, accelerated hematopoietic recovery, and diminished GVHD.³⁵ Use of MSCs in HSCT may have a strong impact for the treatment of GVHD and for tissue repair.

A subset of MSCs that is pluripotent has been isolated by *in vitro* culture.³⁰ These cells, termed multipotent adult progenitor cells (MAPCs), have been shown to be transferred in unfractionated marrow grafts.⁵¹ A recent study demonstrated lymphohematopoietic reconstitution from MAPCs, a finding that would permit what was previously impossible: *in vitro* HSC expansion.⁵⁷ The use of MSCs for cellular and HSC therapy is expanding rapidly and has been recently reviewed.^{36,45}

HEMATOPOIETIC STEM CELL PURIFICATION

Stem Cell Markers

The HSC phenotype has been defined as lineage marker negative (Lin–), Sca-1+, CD34+, c-kit+, Hoechst low, and rhodamine low in mice and baboons.^{5,61,75} Canine CD34+/rhodamine low cells are c-kit+.⁶⁴ CD34 expression was considered the hallmark of the “true” HSC for transplantation until it was demonstrated that CD34– HSCs were capable of successful long-term engraftment.^{26,43} Data suggest CD34 expression reflects activation state and is reversibly expressed by HSCs; thus, HSCs may be variably CD34+ or CD34–.⁵⁵ In dogs, humans, baboons, pigs, cattle, and mice, CD34+Lin– HSCs are capable of long-term multi-lineage hematopoiesis.^{5,12,34,53,61} Anti-canine and anti-bovine CD34 monoclonal antibodies are available for HSC enrichment.^{39,53} The feline CD34 molecule has recently been cloned, but the long-term repopulating ability of feline CD34+Lin– cells has not yet been characterized.⁷⁷

Sources

Bone Marrow

Bone marrow is the classical source of stem cells; HSCs represent approximately 1% of nucleated marrow cells. Studies have shown that marrow-derived HSCs have superior proliferative and reconstitutive ability compared with peripheral blood CD34+ cells, although these differences do not appear to have clinical significance.⁶⁹ Using marrow as a source of HSCs is less demanding technically than the use of peripheral blood, which requires leukapheresis.³⁸

Peripheral Blood

Although “steady-state” (unmobilized) peripheral blood has been shown to be capable of marrow reconstitution, it is ineffective, requiring nearly ten times as many peripheral blood mononuclear cells (PBMCs) as marrow cells. A steady-state PBMC graft comprises only 0.06% HSCs and contains 10 times as many T cells as a marrow graft.³³ However, extravascular HSCs can be mobilized into the blood with the use of hematopoietic growth factors such as granulocyte colony-stimulating factor (G-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF). Mobilization with G-CSF/GM-CSF in humans, mice, and dogs produces a log increase in HSCs so that numbers equal or exceed those in marrow.^{16,33} A recent alternative to G-CSF/GM-CSF is AMD3100, a reversible CXCR4-antagonist that mobilizes HSCs by disrupting binding to stromal-cell-derived factor 1- α . AMD3100 was used successfully in dogs for mobilized PBMC transplants.¹³

There are advantages to the use of mobilized PBMCs over marrow for HSCT. Peripheral blood is more readily accessible and much larger volumes are available; hence many more CD34+ HSCs can be collected than from marrow. The increased proportion of HSCs results in significantly quicker hematologic recovery times with mobilized PBMC transplants compared with BMTs.⁶⁹ A leukapheresis protocol for mobilized canine blood has been described.³⁸

Umbilical Cord Blood

In humans, umbilical cord blood (UCB) contains hematopoietic precursor cells capable of long-term reconstitution. The number of HSCs in UCB is 1–2 logs fewer than in a comparable unit of marrow, but studies have demonstrated UCB to be superior to marrow in reconstituting the marrow progenitor cell population.²¹ Umbilical cord blood grafts show slower hematopoietic recovery, but less GVHD. Greater MHC disparity between donor and host is tolerated for UCB than for PBMCs or marrow.¹¹

In animals, there is little published information on UCB as a source of HSCs.⁶⁷ However, the ontogeny-related functional differences in UCB progenitors are also noted in murine and canine neonatal HSCs. Neonatal HSCs may serve the same purposes in animal models as human UCB transplantation.^{56,64}

TRANSPLANTATION METHODOLOGY

Donor Selection

The risk of graft rejection is correlated to the degree of major histocompatibility complex (MHC) disparity between donors and recipients. Ideal donors for HSCT are matched for both class I and II genes encoded by the MHC gene family. The MHC gene subset that encodes cell surface antigen presenting proteins is known as the leukocyte antigen (LA; see Chapter 101). Leukocyte antigen has been best characterized in

humans and mice. Dog leukocyte antigen (DLA) is fairly well defined, and characterization of feline LA is progressing rapidly.⁷⁹

In allogeneic HSCT, a related LA-matched donor is ideal. A haploidentical donor is a genetically related donor with 1–3 histocompatibility loci mismatches. While GVL is stronger with a haploidentical donor, GVHD and chances for engraftment failure are also greater. In order of suitability, the choices for HSCT donors are: a related LA-matched donor, a haploidentical donor, and an unrelated LA-matched donor. Unrelated LA-matched donors often have other MHC mismatches that are not tested for in routine tissue typing.²⁵

Recipient Conditioning

The two principal aims of recipient conditioning comprise facilitation of HSC engraftment and prevention of GVHD. Requisites for successful engraftment are thought to be the creation of available “space” for donor HSCs within stem cell niches and suppression of the host T cell response to prevent a host versus graft (HVG) response. Currently there is a difference of opinion regarding whether engraftment requires supplanting endogenous HSCs within niches.^{3,7–9,59,63} Historically, the “space” requirement prevailed and pre-transplantation recipient conditioning for both autologous and allogeneic HSCTs predicated myeloablation, usually with total body irradiation (TBI) and/or chemotherapy. Because of the high toxicity associated with myeloablative conditioning, HSCT was only a viable option for young, relatively healthy patients. Hematopoietic stem cell transplantation became more feasible for older patients after dog studies proved that stable chimeras could be achieved with non-myeloablative TBI (2Gy) followed by immunosuppression to control both GVHD and HVG reactions.⁶²

The “space” theory was challenged by dog studies in which successful marrow engraftment was accomplished when irradiation was targeted to lymph nodes, suggesting that TBI functioned to enable engraftment via immune suppression, not by making “space” in marrow niches.⁶³ These findings were refuted in mouse experiments where, in the absence of conditioning, the occupancy of niches did limit donor HSC engraftment.⁷ In support of this, one dog study showed significantly enhanced long-term autologous HSC engraftment with some form of pre-transplantation conditioning.³ However, the debate remains open as to how much and what type of conditioning are necessary, as other groups have achieved successful chimerism with autologous HSCT in dogs and cats without any conditioning.^{9,20,59}

Focused conditioning strategies may avoid toxicities associated with myeloablation. In support of the “space” requirement theory, pre-transplantation conditioning with an anti-HSC (anti-c-kit) antibody in immunodeficient mice permitted robust donor HSC engraftment in the absence of myeloablation.¹⁵ Other strategies to reduce the toxicity of conditioning regimens target the host T cell response. An example is anti-T cell

receptor $\alpha\beta$ conditioning, which resulted in successful engraftment of DLA-matched HSCs in the absence of irradiation.⁶

Post-transplantation, additional immune suppression is required for allogeneic but not autologous HSCT, to prevent HVG and GVH reactions. However, autologous HSCs that are “corrected” via gene therapy may require sustained immune suppression if a novel immunogenic protein is expressed. The incidence of GVHD has been decreased by augmenting a purine inhibitor (e.g. mycophenolate mofetil) with a T cell activation inhibitor (e.g. cyclosporine) or by graft T cell depletion.⁷⁸

Transplantation

The transplantation procedure for HSCs, regardless of source, is similar. Because HSCs home to marrow, there is no need for homotopic transplantation. Grafts are typically administered intravenously at a dose ranging from 10^7 – 10^9 nucleated cells/kg body mass in canine and feline BMTs. Grafts enriched for CD34+ HSCs have been administered in dogs at doses of 10^6 – 10^7 CD34+ cells/kg, similar to the “megadose” HSCTs in humans.²⁸

Outcomes

The HSC graft may either be accepted or rejected by the host. Engraftment is determined by the degree of host chimerism and hematopoietic recovery, and may be either short-term or long-term. The importance of sustained engraftment is defined by the graft’s indication. For marrow reconstitution following intensive chemotherapy for malignancy, short-term engraftment is acceptable if it enables autologous recovery. Conversely, for correction of congenital hematologic disease, long-term engraftment is essential. Because successful engraftment is often associated with GVHD, the overall aim of HSCT is to balance the host and

donor immune responses, enabling engraftment but avoiding GVHD.

COMPLICATIONS OF CONDITIONING AND TRANSPLANTATION

The complications of HSCT are consequences of immune suppression, myeloablation, and GVHD. Susceptible patients acquire infections from infected donor grafts, from the environment, or from reactivation of a latent viral or parasitic infection. Myeloablation complications, apart from immune suppression, primarily concern the propensity for hemorrhage. Today, allogeneic donors are antigenically matched as closely as possible to the recipient, but nearly a third of histocompatible human HSCTs will still develop GVHD. In haploidentical canine HSCTs, median onset for acute and chronic GVHD is 13 and 124 days, respectively.² The clinical signs associated with canine GVHD correlate with the principal organs typically affected, namely gastrointestinal tract, skin, and liver.

The use of TBI for conditioning may result in damage to tissue such as thyroid and pancreas, and may interfere with normal growth in young recipients. In one study, insulin-dependent diabetes mellitus was noted in a feline HSCT patient post-TBI.¹⁹ In dogs, intussusception is a reported potential complication in up to 4.9% of BMT recipients.⁸⁰ The mechanism is not understood, but may be related to post-transplantation immunosuppressive therapy.

CURRENT AND FUTURE APPLICATIONS

Current Treatment Applications

Published uses and outcomes of HSCT in dogs and cats are listed in Table 102.2. This table represents a large sampling of the literature but is not a comprehensive

TABLE 102.2 Therapeutic Applications of HSCT in Dogs and Cats

Application	Species	Outcome	References
Leukemia	Feline	Positive effect	24
Lymphoma	Canine	Positive effect	1, 22, 37, 73
Genetic diseases			
MPS I	Canine, Feline	Positive effect	10, 19, 58
MPS VI	Feline	Positive effect	23
MPS VII	Canine	Positive effect	54
α -Mannosidosis	Feline	Positive effect	71
α -Fucosidosis	Canine	Positive effect	66
GM1-gangliosidosis	Canine	No effect	42
Pyruvate kinase deficiency	Canine	Positive effect	74, 81
CLAD	Canine	Positive effect	4, 60
X-SCID	Canine	Positive effect	20, 28
Glanzmann’s thrombasthenia	Canine	Positive effect	41
Cyclic neutropenia	Canine	Positive effect	76
Chediak-Higashi	Feline	Positive effect	14

^aMPS, mucopolysaccharidosis; CLAD, canine leukocyte adhesion deficiency; X-SCID, X-linked severe combined immunodeficiency.

review. Hematologic malignancies, including lymphoma, are one of the most common indications for human HSCT, but few published reports for this use appear for dogs and cats.^{1,22,24,37,73} Therapeutic transplantation for genetic disease is one of the most published uses of veterinary HSCT because of the invaluable role of animal genetic disease models. The clinical use of autologous “corrected” HSCs for transplantation therapy for genetic disease is in its infancy and many issues remain to be resolved before it can be widely used.²⁷ In humans, HSCT is used to treat aplastic anemia and myelodysplasia, and recently has shown promise for autoimmune disease therapy.^{65,68} Currently there are no published reports for dogs and cats on the use of HSCT for these disorders.

Future Potential: “Transdifferentiation?”

Over the past decade, multiple groups have reported that bone marrow and/or HSCs have the ability to produce non-hematopoietic cells including neurons, liver, and muscle. Some of this “transdifferentiation” was explained by the phenomenon of donor hematopoietic cell fusion with host cells.⁷² However, rare instances of true non-hematopoietic differentiation from donor HSCs have been documented. Some scientists suggest that it may be the MAPC subpopulation in HSC grafts that is responsible for this plasticity.⁵⁷ The field of HSC/MSK plasticity, though contentious, is actively being investigated and is producing interesting results.^{47,52,70}

One of the main impediments to the clinical use of HSCT is the lack of compatible donors. Many veterinary patients are obtained from shelters or pet stores; thus related donors are not available. The overall expense, complications of immune suppression, lack of widely available donor typing, and intensive supportive care measures are all prohibitive factors to its use outside of the academic arena. Finally, the ethics of HSCT in veterinary patients cannot be dismissed. In a patient incapable of informed consent, the consequences of HSCT must be carefully weighed against those of the disease process and alternative therapeutic strategies.

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Clinical Use of Hematopoietic Growth Factors

STEVEN E. SUTER

Erythropoietin

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Acronyms and Abbreviations

BMT, bone marrow transplantation; CRF, chronic renal failure; EPO, erythropoietin; FDA, Food and Drug Administration; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; Hct, hematocrit; HGF, hematopoietic growth factor; IL, interleukin; M-CSF, macrophage colony-stimulating factor; PBPC, peripheral blood progenitor cell; RBC, red blood cell; rcEPO, recombinant canine erythropoietin; rfEPO, recombinant feline erythropoietin; rhEPO, recombinant human erythropoietin; SCF, stem cell factor; TBI, total body irradiation; TPO, thrombopoietin.

The hematopoietic system is an exquisitely controlled hierarchical tissue, with multipotent stem cells residing mainly in the bone marrow eventually becoming nonreplicative mature hematopoietic cells that circulate in the peripheral blood. The endogenous elaboration of hematopoietic growth factors (HGFs), stimulatory glycoproteins that affect the growth and differentiation of bone marrow-derived cells from these stem cells, drives this process. Some of these HGFs exert their effects on multiple cell lineages, while others are cell lineage specific. The availability of these HGFs has become invaluable in human medicine, with marked clinical advancements in bone marrow transplantation (BMT) and chemotherapy-induced myelosuppression, as well as in a variety of other disorders.

Several canine and feline HGFs have been cloned, although none are commercially available for clinical applications. The use of recombinant human HGFs is relatively commonplace in veterinary medicine but still considered strictly an extra label application. The following brief review will discuss the most clinically relevant HGFs used in veterinary medicine.

ERYTHROPOIETIN

Background

Erythropoietin (EPO), a red blood cell (RBC) lineage-specific hormone, is produced mainly by juxtatubular interstitial cells of the renal cortex, with a small amount being produced by the liver. Erythropoietin cooperates with various other HGFs (interleukin-3 [IL-3], stem cell factor [SCF], thrombopoietin [TPO]) in the development of colony-forming unit-erythroid cells in the bone marrow from a common myeloid precursor. Erythropoietin promotes erythroid progenitor cell survival through apoptosis evasion via up-regulation of the antiapoptotic protein BCLX_L¹⁶ and by reducing the level of cell-cycle inhibitors. The end result is increased production of proerythroblasts and release of more RBCs into the circulation. It is now recognized that hypoxia, the most potent stimulus of EPO production, increases the activity of the primary EPO transcription factor, hypoxia-inducible factor 1 α .⁴⁰

In 1989 EPO became the first HGF to become commercially available for clinical use in the United States.

Recombinant human EPO (rhEPO, epoetin alpha) (Epogen, Amgen, Thousand Oaks, CA; Procrit, Ortho Biotech, Bridgewater, NJ), is approved by the US Food and Drug Administration (FDA) for the treatment of anemia associated with chronic renal failure (CRF), zidovudine (Retrovir) therapy in patients infected with human immunodeficiency virus, and chemotherapy for nonmyeloid malignancies, as well as anemia in patients scheduled for elective, noncardiac, nonvascular surgery. In 2001 darbepoetin alpha (Aranesp, Amgen), a hyperglycosylated molecule based on the protein backbone structure of human EPO, was approved by the FDA to allow a new option for treatment of anemia of CRF. The increased level of glycosylation allows a longer duration (up to three times longer than EPO) of the drug in the circulation.¹¹ Subsequently, darbepoetin alpha was approved for the treatment of chemotherapy-induced anemia in patients with nonmyeloid malignancies.

Use in Veterinary Medicine

Similar to the human experience, EPO most likely plays an important role in the pathogenesis of anemia in companion animals. The suppressed rate of RBC formation caused by lack of renal production of EPO is a major problem in the treatment of patients with CRF.³⁶

Fortuitously, the structure of EPO is well conserved across species lines. When given to dogs and cats with CRF, rhEPO causes a dose-dependent increase in the hematocrit (Hct) and corrects anemia.⁶ A transient, moderate reticulocytosis is initially seen and the bone marrow myeloid:erythroid ratio decreases, illustrating the increased erythropoietic response. Depending on the dose given and the initial Hct, correction of the Hct to low normal takes from 2 to 8 weeks. When RBC production is noted, it usually results in a 1% increase per day in the dog's or cat's Hct.⁶

Dosing schedules for EPO have not been standardized for veterinary patients. Both intravenous and subcutaneous routes of administration of rhEPO appear effective, although plasma concentrations persist longer after subcutaneous administration, which allows lower total doses to be given. Starting doses of 50–150 U/kg given subcutaneously three times per week are currently recommended for dogs (100 U/kg in cats) with weekly monitoring continued until a target Hct is achieved (30–35% in dogs and 25–30% in cats).^{3,15,26,50} Thereafter, the dosing interval should be decreased to twice weekly. To maintain RBC count and Hct in the target range, most animals require 50–100 U/kg one to two times weekly, although the dosage required varies significantly among treated individuals. Therefore, periodic monitoring of the Hct is required for proper adjustment of the dose or dosing interval or both. Pharmacological activity in dogs has also been demonstrated using a mucoadhesive tablet containing 100 U/kg rhEPO.⁵¹ Supplementation with oral ferrous sulfate (100–300 mg/day for dogs and 50–100 mg/day for cats) is recommended for all patients treated with rhEPO due to the high demand for iron associated with stimulated

erythropoiesis, even if the pretreatment transferrin saturation is normal.²¹

Reported adverse reactions to EPO include transient cutaneous or mucocutaneous reactions with or without fever (rare and self-limiting upon cessation of rhEPO), hypertension, seizures, polycythemia, vomiting, discomfort at the site of injection, and, most significant clinically, the development of neutralizing antibodies and RBC aplasia. Approximately 20–30% of rhEPO treated animals develop neutralizing antibodies against the foreign protein, leading to RBC aplasia.^{6,15} Although anti-rhEPO antibody titers may decline after discontinuation of treatment and pretreatment levels of erythropoiesis can be attained, the severe anemia seen before treatment remains since these levels are not sufficient to maintain adequate RBC numbers. Due to the possible long-term prevalence of anti-rhEPO antibodies in treated dogs and cats, administration of rhEPO is generally not recommended until the anemia of CRF is negatively impacting the animal's quality of life.

More recently, the clinical safety and efficacy of recombinant canine EPO (rcEPO) was investigated in dogs with CRF and rhEPO-induced RBC aplasia.^{37,39} Recombinant canine EPO stimulated RBC production in dogs with nonregenerative anemia secondary to CRF without causing the profound RBC aplasia that can occur in rhEPO-treated dogs, although rcEPO was not as effective in restoring RBC production in dogs that had previously developed rhEPO-induced RBC aplasia.

Recombinant feline EPO (rfEPO) has also been produced in Chinese hamster ovary cells.^{1,38} The biological activity of rfEPO was broadly equivalent to rhEPO, with the median Hct and absolute reticulocyte count in cats with CRF treated with rfEPO increasing significantly during the first 3 weeks of treatment. Unfortunately, development of RBC aplasia during treatment with rfEPO was also seen, most likely due to the development of antibodies against a Chinese hamster ovary antigen that cross-reacts with feline EPO.

Darbepoetin alfa has also been anecdotally used at several veterinary institutions to correct canine and feline CRF-induced anemia, although published data documenting its efficacy are lacking.

GRANULOCYTE-COLONY STIMULATING FACTOR

Background

Granulocyte-colony stimulating factor (G-CSF) is an 18.8 kDa glycoprotein produced by bone marrow stromal cells, monocytes/macrophages, and endothelial cells. Granulocyte-colony stimulating factor binds primarily to granulocyte progenitor cells in the bone marrow, stimulating the proliferation and maturation of neutrophil precursors. At higher doses, G-CSF can act in a multilineage fashion, stimulating the differentiation of several other hematopoietic progenitors. Granulocyte-colony stimulating factor also promotes

neutrophil migration across the vascular endothelium and primes neutrophils for cell killing. FDA approved, commercially available human G-CSF includes: lenograstim (Granocyte, Chugai Pharma, USA), filgrastim (Neupogen, Amgen), and pegylated filgrastim (Neulasta, Amgen).

Human G-CSF was cloned in 1986 and approved by the FDA in 1991 to protect cancer chemotherapy patients. In humans, G-CSF administration causes a dose-dependent rise in neutrophils, as well as a moderate increase in monocytes. Granulocyte-colony stimulating factor is now routinely given to human patients undergoing chemotherapy to help prevent episodes of severe neutropenia; G-CSF may also be beneficial in the treatment of established afebrile or febrile neutropenia by reducing the length, and thus expense, of hospitalization stays.⁵

Granulocyte-colony stimulating factor has also been used in humans for the treatment of cyclic neutropenia, an inherited disease characterized by recurrent episodes of neutropenia occurring every 20–24 days. Following rhG-CSF administration the mean neutrophil counts increase, and the severity of neutropenia associated with the cycles decreases. Granulocyte-colony stimulating factor does not completely eliminate the neutrophil cycling or the clinical signs associated with disease, suggesting a defect in receptor signaling, rather than a paucity of G-CSF production.

Another important setting in human medicine where G-CSF is routinely used is in association with BMT. The administration of G-CSF after high-dose chemotherapy and autologous BMT improves the rate of peripheral blood neutrophil recovery, reducing the morbidity and mortality associated with febrile neutropenic episodes. Granulocyte-colony stimulating factor also mobilizes hematopoietic CD34+ progenitors for collection prior to myeloblastic therapy. After treatment with G-CSF for 5 days, high concentrations of CD34+ progenitor cells are found in the peripheral blood which can be collected by leukapheresis and administered to a patient after high-dose chemotherapy or total body irradiation (TBI) in place of marrow. “G-priming” of donors is now standard practice in human transplant protocols. Other uses of G-CSF include treating patients with myelodysplasia and acquired immunodeficiency syndrome-associated bone marrow aplasia.

Use in Veterinary Medicine

Canine G-CSF has been cloned (rcG-CSF, Amgen) and produced synthetically for research purposes but is not commercially available. rcG-CSF transiently increases neutrophil and macrophage numbers in dogs and cats. At a dose of 5 µg/kg/day, mean neutrophil counts in healthy dogs increased to 26,330/µL within 24 hours of the first injection of rcG-CSF and reached a maximum of 72,125/µL by day 19.³³ A similar effect on neutrophil counts was noted in cats following administration of rcG-CSF at a dose of 5 µg/kg/day.³⁴

Mirroring the human experience, rcG-CSF is able to reduce the duration and severity of chemotherapy-

induced myelosuppression.³⁵ Five of 10 dogs administered mitoxantrone (5 µg/m² IV) were also given rcG-CSF (5 µg/kg/day SC beginning 1 day after chemotherapy and continued for 20 days), resulting in median neutrophil counts dropping below 3,000/µL for only 2 days in comparison to 5 days for those dogs not receiving rcG-CSF. Additionally, four of the five dogs that did not receive rcG-CSF developed severe neutropenia (<1,500/µL) while none of the rcG-CSF treated dogs did.

Cyclic hematopoiesis has also been identified in gray collie dogs.⁵² Dogs with this disease respond similarly to humans given rhG-CSF, although the development of neutralizing antibodies and subsequent prolonged neutropenia with chronic rhG-CSF usage occurs.²² This problem was negated by giving rcG-CSF at a dose of 1–2.5 µg/kg SC every 12 hours.²³

The use of G-CSF in the setting of TBI and BMT has also been reported in the veterinary literature. Granulocyte-colony stimulating factor leads to complete bone marrow reconstitution within 21 days after lethal TBI⁴⁴ and can also mobilize bone marrow CD34+ progenitor cells for peripheral blood stem cell transplantation.⁹ Granulocyte-colony stimulating factor can also “prime” dogs before marrow harvesting in the setting of malignant lymphoma. rcG-CSF increased donor peripheral blood mononuclear cell numbers (containing CD34+ progenitor cells) for harvesting via leukapheresis with subsequent allogeneic BMT to an unrelated dog with stage V(b) T-cell multicentric lymphoma who received 8Gy TBI.²⁵ The transplanted dog maintained a complete remission for at least 58 weeks after BMT. rcG-CSF has also been used to successfully “prime” dogs before bone marrow harvesting via bone marrow aspiration.¹³ The reader is referred to a comprehensive review of the use of hematopoietic cytokines, including G-CSF, in the BMT setting,²⁴ as well as an original study describing the use of leukapheresis, both with and without rcG-CSF “priming”, to harvest peripheral blood progenitor cells (PBPCs) from dogs.²³

GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR

Background

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a variably glycosylated (10–30 kDa) protein produced by fibroblasts, endothelial cells, monocytes, and T lymphocytes that has a variety of biological activities. These include prolonging the survival of hematopoietic progenitors and mature neutrophils, eosinophils, and macrophages, stimulating the proliferation of progenitor cells, and enhancing the functional capacity of the mature cells.

The FDA approved formulations of rhGM-CSF (sargramostim) are Leukine (Bayer HealthCare Pharmaceuticals, USA) and Prokine (Amgen). Both are approved for use in human patients more than 55 years

of age with acute myelogenous leukemia following induction chemotherapy. Leukine is also approved for use in a number of hematopoietic stem cell transplant settings, including BMT failure or engraftment delay, myeloid reconstitution after autologous and allogeneic BMT, and mobilization and following transplantation of autologous PBPCs. The reported side effects of rhGM-CSF in human patients include bone pain, fever, and flu-like symptoms.

Use in Veterinary Medicine

Recombinant canine GM-CSF (rcGM-CSF) has been cloned,³⁰ although it is not commercially available for clinical use. rcGM-CSF given to normal dogs caused a significant increase in circulating neutrophils and monocytes, whereas the effect on circulating eosinophils was more variable.³¹ rhGM-CSF is active in dogs,²⁸ inducing neutrophilia and eosinophilia, although it produced variable results in dogs with chemotherapy-induced myelosuppression.⁴⁵ This is most likely because rcGM-CSF is only 70% homologous with the human protein, and, therefore, higher doses of rhGM-CSF may be necessary to achieve the same effect as rcGM-CSF. rhGM-CSF also prevents recurrent neutropenia in gray collie dogs with cyclic hematopoiesis, although most dogs developed antibodies to the protein within 2–3 weeks of treatment and hematopoietic cycling persisted.¹⁷

Importantly, GM-CSF also has the ability to activate monocytes and macrophages, increasing their ability to recognize and destroy malignant cells.¹⁴ DNA plasmid vectors containing the coding sequence for GM-CSF can deliver GM-CSF directly to tumors, which theoretically allows the local recruitment and activation of macrophages. rhGM-CSF cDNA was cloned into a xenogeneic expression vector and injected intramuscularly biweekly for a total of four vaccinations into three dogs diagnosed with oral malignant melanoma. In this same study, nine dogs were injected with both rhGM-CSF and huTyrosinase.² The authors reported a median survival time of 148 days for dogs receiving rhGM-CSF alone and >402 days for dogs receiving both rhGM-CSF and huTyrosinase. Another anti-malignancy strategy is to remove the primary tumor or metastatic lesion, culture the tumor cells *in vitro*, and transfect them with a eukaryotic vector encoding GM-CSF or use GM-CSF transfected cell lines established from primary tumors.^{12,18} There are currently a wide variety of human clinical trials examining the safety and efficacy of both viral and non-viral vectors encoding GM-CSF for the treatment of many types of solid and hematopoietic malignancies.

THROMBOPOIETIN

Background

Thrombopoietin, a 35 kDa glycoprotein produced primarily in the liver, is an important regulator of throm-

bopoiesis via its role as a late-acting megakaryocyte maturation factor. It also stimulates hematopoietic progenitor cells (CD34+) to undergo proliferation and differentiation into megakaryocytic colonies and synergizes with SCF and IL-3 to stimulate hematopoietic progenitor cell division and differentiation. The receptor for TPO is encoded by the proto-oncogene *c-mpl*. Neumega (oprelvekin, Wyeth, USA) is a synthetic 19 kDa version of human IL-11 with FDA approval for use to prevent severe thrombocytopenia and reduce the need for platelet transfusions following myelosuppressive chemotherapy. Nplate (romiplostim, a thrombopoietin analog, Amgen) is a commercially available peptibody protein with FDA approval for the treatment of thrombocytopenia in adults with chronic immune thrombocytopenic purpura.

In the setting of BMT, TPO can stimulate enhanced platelet recovery and improve the recovery of other hematopoietic lineages after BMT, and act synergistically with G-CSF to accelerate neutrophil recovery in mice after radiation or chemotherapy. The reader is referred to two recent review articles for a more thorough discussion of TPO and the development of TPO mimetics.^{20,49}

Use in Veterinary Medicine

Theoretically, mirroring the human experience, TPO or any TPO mimetics could be useful in veterinary medicine for any disease process causing thrombocytopenia. Additionally, TPO may be useful in the setting of chemotherapy-induced thrombocytopenia and for hematopoietic recovery after BMT. Feline, but not canine, TPO has been cloned.²⁷ However, there are no reports in the literature describing use of rTPO, Nplate, or Neumega in either research or clinical settings in companion animals.

INTERLEUKIN-3

Background

Interleukin-3 is a 25 kDa glycoprotein produced primarily by T cells that acts mainly on CD34+ hematopoietic progenitor cells. Interleukin-3 promotes the survival and expansion of these cells and also, to a lesser extent, produces the same effects on committed progenitors of the granulocyte-macrophage, erythroid, eosinophil, basophil, megakaryocyte, and mast cell lineages. Interleukin-3 also enhances myeloid cell functions. Clinically, IL-3 leads to multilineage expansion of myeloid cells, including platelets, neutrophils, and monocytes, when used alone or in combination with rhGM-CSF. Therefore, IL-3 may be useful to treat chemotherapy-induced myelosuppression, although signs of toxicity, including fever, fatigue, and headaches are common. The reader is referred to a recent review article describing the role of IL-3 in hematopoietic cell development.⁸

Use in Veterinary Medicine

Canine IL-3, which was cloned in 2001,¹⁹ has approximately 37% amino acid homology to the human protein. This may explain the lack of efficacy of the human protein (rhIL-3) in increasing neutrophil or macrophage numbers when administered to dogs. rhIL-3 failed to correct recurrent neutropenia in gray collie dogs with cyclic hematopoiesis, although it did stimulate eosinophilia.¹⁷

STEM CELL FACTOR

Background

Stem cell factor is a 30kDa glycoprotein produced by bone marrow stromal cells, fibroblasts, and endothelial cells. The SCF receptor is encoded by the proto-oncogene *c-kit* that is expressed on hematopoietic progenitor cells, mast cells, and melanocytes.

Stem cell factor acts synergistically with many other HGFs to promote the production of lymphoid, myeloid, and erythroid lineages from hematopoietic progenitor cells.⁴⁸ Therefore, SCF has potential benefit in a number of clinical settings involving either hematopoietic precursor damage or defects and in the setting of BMT. Administration of rhSCF to humans results in significantly increased hematopoietic progenitor cells in the bone marrow and blood. Stem cell factor can enhance the proliferation of bone marrow cells derived from patients with aplastic anemia when used with other HGFs. Other uses include the administration of SCF after BMT or chemotherapy to enhance hematopoietic recovery.

Use in Veterinary Medicine

Canine SCF was cloned in 1992 and shares approximately 85% amino acid homology to the human protein.⁴² Feline SCF, which has a high degree of homology at both the nucleic acid and protein level to other species, has also been cloned.¹⁰ The vast majority of veterinary literature describes the use of rcSCF in veterinary patients in the settings of hematopoietic recovery after BMT and canine mast cell disease.^{46,47} rcSCF supports the survival of hematopoietic progenitors in long-term marrow culture³² and acts synergistically with rcG-CSF to decrease the time to hematopoietic recovery in dogs after TBI.⁴¹ rcSCF, either alone or in combination with rcG-CSF, also significantly increases the level of PBPCs and stem cells.^{9,43} rcSCF logically enhances IgE-mediated histamine and TNF- α release in canine mast cells since mature mast cells express *c-kit*.⁴ rcCSF, alone or in combination with rcG-CSF, has also been used to treat gray collies with cyclic neutropenia.⁷

CONCLUSIONS

Hematopoietic growth factors have significant potential in the treatment of veterinary patients in a wide variety

of settings. The greatest benefit associated with the use of HGFs is for those patients with profound blood cell dyscrasias secondary to chemotherapy administration or BMT. Mirroring the human experience, the use of HGFs in conjunction with the administration of chemotherapies that are toxic to the bone marrow may provide an arena to allow the safe exploration of increased anti-cancer dose intensity which could result in increased survival times while improving quality of life. In addition, as more reports of canine BMT are published, HGFs will most likely become an important part of these protocols in an effort to increase our ability to harvest hematopoietic progenitor CD34+ cells and aid in post-BMT hematologic recovery. Unfortunately, until recombinant canine and/or feline HGFs become affordable commercially available products, a veterinarian's only option is to use recombinant human proteins which are, quite often, expensive, ineffective, and have the potential to lead to the development of debilitating neutralizing antibodies with chronic use.

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SECTION IX

Species Specific Hematology
Rose E. Raskin and K. Jane Wardrop

Normal Hematology of the Dog

THERESA E. RIZZI, JAMES H. MEINKOTH, and KENNETH D. CLINKENBEARD

Erythrocytes	Normal Morphology
Morphology	Quantitative Parameters
Quantitative Parameters	Platelets
Hemoglobin, hematocrit and red blood cell count	Morphology
Mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration	Quantitative Parameters
Red cell distribution width	Bone Marrow
Reticulocytes	Morphology
Leukocytes	Erythroid series
	Granulocytic series
	Megakaryocytic series
	Quantitative Parameters

Acronyms and Abbreviations

Hct, hematocrit; Hgb, hemoglobin; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; MCV, mean cell volume; N:C ratio, nuclear:cytoplasmic ratio; nRBC, nucleated red blood cell; RBC, red blood cell; RDW, red cell distribution width; WBC, white blood cell.

ERYTHROCYTES

Morphology

The canine erythrocyte is a biconcave disc, approximately 7 μm in diameter. The biconcave shape is more pronounced in the dog than in other domestic animals, giving their red cells a clearly visible central pallor when viewed in the monolayer of well made blood films (Fig. 104.1). This prominent central pallor, which normally encompasses approximately the central one-third to one-half of the diameter of the cell, makes recognition of spherocytes possible in this species. Central pallor may be lost at the edges of a smear or in very thin smears, where sheer forces distort normal morphology (Fig. 104.2).

Canine erythrocytes normally display only mild anisocytosis and poikilocytosis. Echinocyte formation may be an artifact related to smear preparation (i.e. crenation), but has also been reported in certain disease states.⁴⁴ Clear, refractile areas (artifacts) within red cells may be seen if the blood smear is not adequately dried before staining. Stain precipitate may sometimes adhere to erythrocytes and must not be confused with parasites (Fig. 104.3).

When blood smears are stained with Romanowsky stains, such as Wright's stain, polychromatophilic (blue staining) cells may be seen occasionally, but in health these comprise less than 1% of the cells (Fig. 104.4). These represent erythrocytes recently released from the bone marrow and are recognized as reticulocytes when blood is stained with certain supravital stains, such as new methylene blue. Canine reticulocytes are mostly of the aggregate type, containing one or more large, distinct clumps of blue staining material (Fig. 104.5). In dogs, polychromasia noted on Romanowsky stained smears correlates well with reticulocytosis, although accurate, reproducible evaluation of polychromasia requires more microscopy experience.¹⁹

Rare nucleated red blood cells (nRBCs) and Howell-Jolly bodies can be seen in blood smears from normal dogs. Nucleated RBCs may be more numerous in newborn pups, but numbers decrease rapidly within the first week of life and reach adult levels by approximately 1–2 months of age.^{11,39} Hemoglobin (Hgb) crystals are square to rectangular structures which stain similarly to or slightly darker than Hgb and often deform the RBC that contains them. Low numbers (1–10 per slide) of Hgb crystals have been reported in blood smears of clinically normal dogs and dogs less than 3

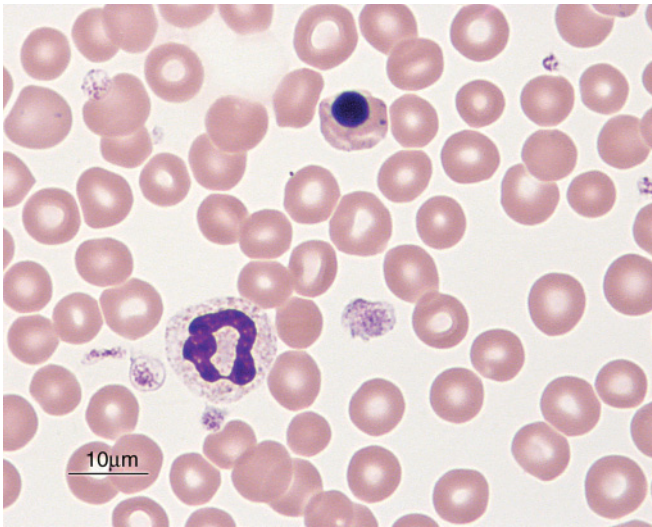


FIGURE 104.1 Canine erythrocytes showing normal central pallor. A single mature neutrophil is present. The granules in this neutrophil stain a faint pink. A single metarubricyte is present at the top. Wright-Giemsa stain.

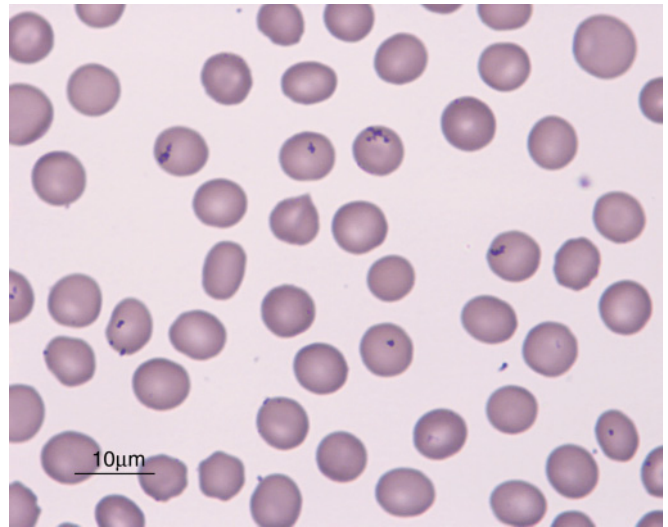


FIGURE 104.3 Stain precipitate adhered to erythrocytes. Wright-Giemsa stain.

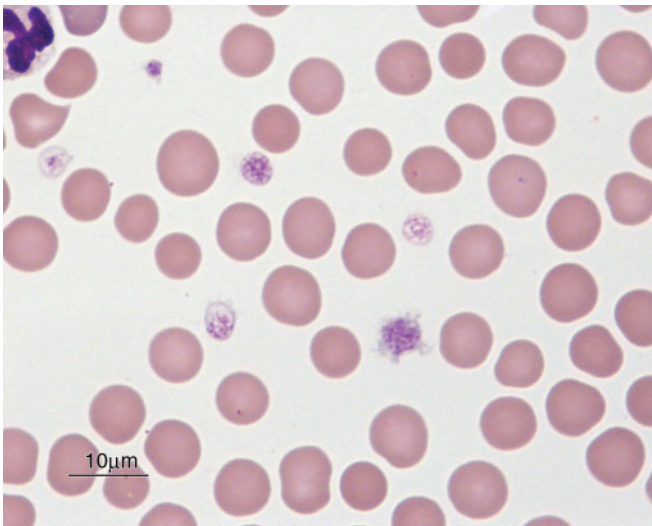


FIGURE 104.2 Different field from the same blood film. The erythrocytes have lost their pallor due to sheer artifact. Wright-Giemsa stain.

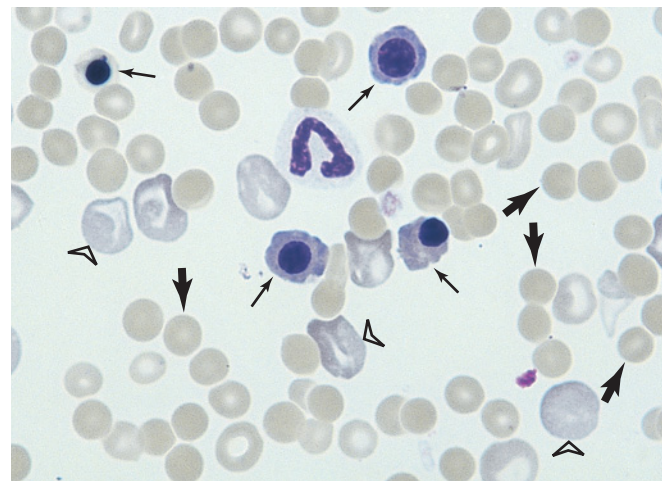


FIGURE 104.4 Blood smear from a dog with an immune-mediated hemolytic anemia. Many large, polychromatophilic erythrocytes are present (arrowheads). Four nucleated red cells (rubricytes and metarubricytes) are also present (thin arrows). Many of the mature erythrocytes are spherocytes (thick arrows). Wright-Giemsa stain; 100× objective. (Courtesy of Oklahoma State University Clinical Pathology Teaching Files.)

months of age.²³ Their formation may be an in-vitro artifact associated with delayed sample processing.² They have not been associated with abnormal Hgb molecules.

Quantitative Parameters

Table 104.1 provides historically published reference intervals for erythrocyte parameters of adult dogs. Reference intervals are designed to include values

found in the majority of “normal” animals and will vary according to analyzer, population used to collect the data and the definition of “normal.” Many published reference intervals for canine hematologic values are derived from research animals and represent a single breed raised under confinement conditions. Such intervals may be narrower than would be applicable to a general patient population in which breed, age and environmental variables are present. Alternatively, reference intervals that are derived from shelter dogs or

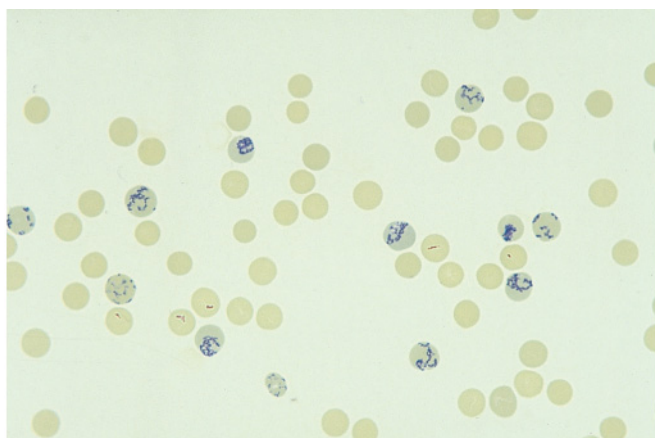


FIGURE 104.5 Canine reticulocytes. Most canine reticulocytes have large aggregates of blue staining precipitate. New methylene blue stain; 100× objective. (Courtesy of Oklahoma State University Clinical Pathology Teaching Files.)

TABLE 104.1 Hematologic Reference Intervals for Normal Dogs^a

Parameter	Units	Reference Interval
Erythrocytes	×10 ⁶ /μL	5.5–8.5
Hemoglobin	g/dL	12.0–18.0
PCV	%	37–55
MCV	fL	60–77
MCHC	%	32–36
Reticulocytes	%	0.0–1.5
Reticulocytes	/μL	<70,000
Leukocytes	/μL	6,000–17,000
Neutrophils	/μL	3,000–11,500
Bands	/μL	0–300
Lymphocytes	/μL	1,000–4,800
Monocytes	/μL	150–1,350
Eosinophils	/μL	100–1,250
Basophils	/μL	Rare
Platelets	/μL	200,000–500,000
Mean platelet volume	fL	6.7–11.1 ^b 3.9–6.1 ^c

^aData from reference 17

^bData from reference 26

^cData from reference 45

“clinically healthy” animals brought to a veterinary clinic may be inappropriately wide because of subclinical disease, nutritional status, and physiologic or environmental influences (e.g. fear associated with being in a strange environment and phlebotomy). Common, clinically significant sources of variability are discussed below.

Hemoglobin, Hematocrit, and Red Blood Cell Count

All three of these measurements of red cell mass are interrelated and, in the absence of marked alterations of RBC size and Hgb concentration, tend to parallel

each other. Hematocrit (Hct), the percentage of blood volume comprising erythrocytes, can be determined either as a spun Hct by centrifugation (“packed cell volume;” PCV) or a calculated Hct based on the mean corpuscular volume (MCV) and RBC count. Most current automated hematology analyzers utilize calculated Hct, although spun Hct is still commonly determined in practice and when artifactual calculated Hct values are suspected.

The RBC mass of neonatal animals varies significantly from that of adults (Table 104.2). At birth, Hgb, Hct and the RBC count are near adult values but decline rapidly over about the first 2 months of life. The magnitude of the drop is clinically significant, with Hct values reaching a nadir in the high 20s to low 30s.³⁹ After this, values start to increase, generally reaching adult levels by approximately 6 months to 1 year of age.^{1,4,11,15}

Some authors have found a gender affect on RBC mass. One study of Beagles showed that males had a slightly higher Hgb concentration than females (16g/dL vs. 15.6g/dL).²⁷ The magnitude of this change is not likely to be clinically significant as all values are well within the standard reference intervals for adult dogs. Pregnant females have significant changes during gestation. In one study, the Hct of pregnant dogs fell from a baseline of 53% to 32% at term.⁴ Numerous studies have shown that Greyhound dogs generally have higher values related to RBC mass than other dogs, with Hcts up to about 65% commonly reported in clinically healthy animals.^{9,11,20,34,38} This effect is age related, with values for parameters of RBC mass being higher than non breed-specific reference intervals by approximately 9–10 months of age.³⁸

Mean Corpuscular Volume, Mean Corpuscular Hemoglobin and Mean Corpuscular Hemoglobin Concentration

These RBC indices characterize the size and hemoglobinization of the red cell population. In the dog, fetal red cells are larger than those of adults. The MCV of umbilical cord blood is 100–110fL and in newborn pups is 95–100fL, but rapid replacement of fetal erythrocytes following birth brings this value to adult levels by 2–3 months.^{24,39} Breed variations of erythrocyte size have been reported. Some lines of toy and miniature poodles have an inherited macrocytosis with abnormal erythropoiesis.^{5,37} These animals have RBC counts that are below standard reference intervals, but due to the large size of the RBCs and increased Hgb content, their Hct and Hgb are normal. The reported MCV of affected animals ranges from 84.5 to 106.7fL (compared to 60–77fL in unaffected animals). Japanese Akita and Shiba breeds have microcytic RBCs with MCV ranging from 55 to 65fL.^{7,13}

Red Cell Distribution Width

The red cell distribution width (RDW) represents the size variability of individual erythrocytes analyzed.

TABLE 104.2 Blood Values in Normal Beagles to 2 Months of Age^a

	Age				
	0–3 days	14–17 days	28–31 days	40–45 days	56–59 days
Number of dogs	46	46	48	44	42
RBCs ($\times 10^6/\mu\text{L}$)	4.8 ± 0.8	3.5 ± 0.3	3.9 ± 0.4	4.1 ± 0.4	4.7 ± 0.4
Hemoglobin (g/dL)	15.8 ± 2.9	9.9 ± 1.1	9.6 ± 0.9	9.2 ± 0.7	10.3 ± 0.9
PCV (%)	46.3 ± 8.5	28.7 ± 2.9	28.4 ± 2.5	28.3 ± 2.3	31.4 ± 2.4
MCV (fL)	94.2 ± 5.9	81.5 ± 3.3	71.7 ± 3.5	68.2 ± 2.6	65.8 ± 2.3
MCH (pg)	32.7 ± 1.8	28.0 ± 2.0	24.3 ± 1.6	22.4 ± 1.0	21.8 ± 1.2
MCHC (%)	34.6 ± 1.4	34.3 ± 1.6	33.5 ± 1.4	32.4 ± 1.7	32.6 ± 1.8
nRBC/100 WBC	7.2 ± 6.7	2.4 ± 3.8	1.1 ± 1.5	0.6 ± 0.9	0.1 ± 0.4
Reticulocytes (%)	6.5	6.7	5.8	4.5	3.6
WBCs/ μL	$16,800 \pm 5,700$	$13,600 \pm 4,400$	$13,900 \pm 3,300$	$15,300 \pm 3,700$	$15,700 \pm 4,400$
Absolute number of WBCs/ μL					
Band neutrophils	600 ± 500	200 ± 200	100 ± 200	200 ± 200	300 ± 300
Segmented neutrophils	$9,200 \pm 6,600$	$6,900 \pm 3,100$	$6,800 \pm 2,000$	$7,400 \pm 2,400$	$8,500 \pm 2,900$
Lymphocytes	$3,700 \pm 2,300$	$4,900 \pm 1,700$	$5,400 \pm 1,600$	$6,100 \pm 1,900$	$5,000 \pm 1,500$
Monocytes	$1,400 \pm 1,300$	$1,100 \pm 600$	$1,100 \pm 600$	$1,300 \pm 600$	$1,400 \pm 700$
Eosinophils	400 ± 400	500 ± 500	400 ± 400	300 ± 300	400 ± 400
Platelets/ μL ^b	302,000	290,000	287,000	321,000	411,000
Myeloid:erythroid ratio ^b	1.6:1	1.7:1	1.7:1	1.8:1	1.4:1

^aData from Shifrine M, Munn SL, Rosenblatt LS, et al. Hematologic changes to 60 days of age in clinically normal beagles. *Lab Anim Sci* 1973;23:894–898.

^bData from Earl FL, Melveger BE, Wilson RL. The hemogram and bone marrow profile of normal neonatal and weanling Beagle dogs. *Lab Anim Sci* 1973;23:690–695. Values were approximated for various age groups shown here; 5 males and 5 females were studied in each group.

Numerically it represents the coefficient of variation of RBC volumes and can be graphically represented on a histogram. Increases in RDW indicate increased anisocytosis of the erythrocyte population and may be more sensitive than the MCV in detecting early changes in erythrocyte size.³¹

Reticulocytes

Reticulocytes are released from the canine marrow in a cyclic manner with a periodicity of approximately 14 days.³⁰ Reference intervals will vary between laboratories based on methodology employed and reference population used; however, adult dogs generally have less than 1.0% reticulocytes in peripheral blood or an absolute reticulocyte count of less than 70,000/ μL . Neonatal animals have significantly higher reticulocyte counts, a manifestation of increased erythropoiesis needed to keep up with a rapidly expanding vascular volume. Reticulocyte counts of up to approximately 10% can be seen in dogs during the first 2 months of life.^{10,11} This number decreases to adult levels by approximately 5–6 months of age.

LEUKOCYTES

Normal Morphology

Neutrophils are the most abundant leukocyte in the peripheral blood of normal dogs (Fig. 104.6). The mature neutrophil has an elongated nucleus which contains several distinct lobes separated by constricted areas.

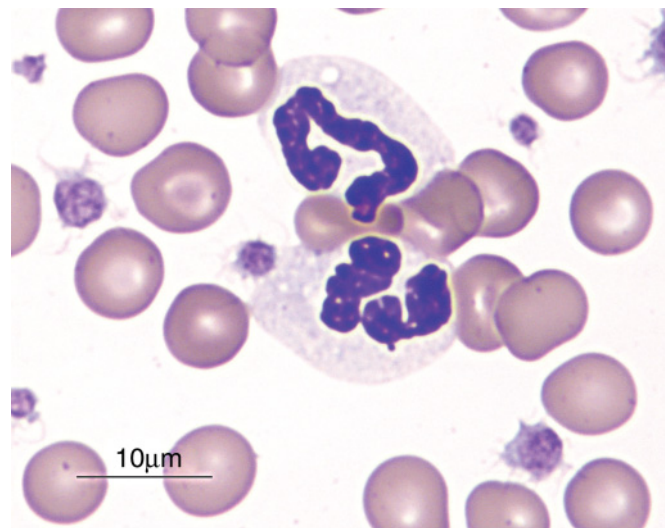


FIGURE 104.6 Two mature neutrophils showing mature chromatin and segmented nuclei. Wright-Giemsa stain.

These constricted areas are usually simply narrowing of the nuclear material, but may form thin filamentous structures. The nucleus stains dark purple and has a clumped chromatin pattern consisting of many areas of densely staining heterochromatin. Cytoplasm is typically clear to slightly eosinophilic. Neutrophil granules in the dog are typically indiscernible, but may be faintly eosinophilic. Immature, or band shaped, neutrophils may be present in low numbers in normal animals.

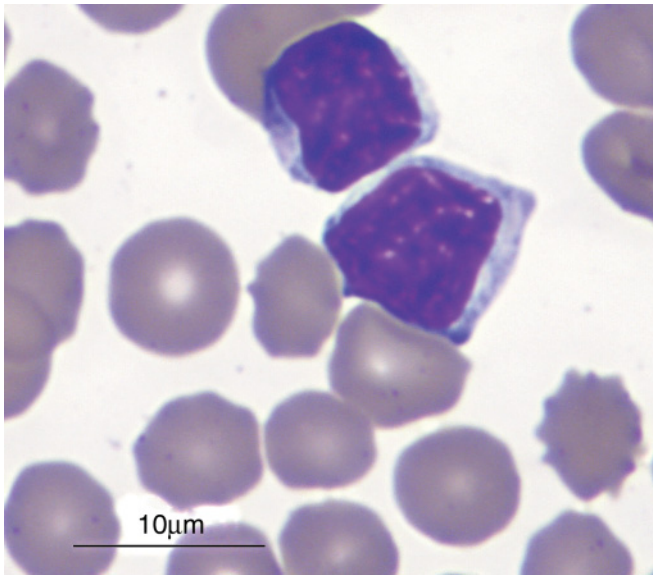


FIGURE 104.7 Two small lymphocytes with dark chromatin and scant cytoplasm. Wright-Giemsa stain.

These cells contain elongated nuclei that lack the distinct lobation that characterizes mature cells. The nucleus should have generally parallel sides, lacking any discrete nuclear constrictions. The nuclear chromatin of a band cell is generally less condensed and lighter staining than that of a mature cell. Cytoplasm is similar to that of mature neutrophils.

Lymphocytes in the peripheral blood vary in size, with small lymphocytes being most abundant (Fig. 104.7). Small lymphocytes have densely staining nuclei which range from generally round with a flattened side to oval to slightly indented. Nuclear chromatin is densely clumped. Small lymphocytes have only scant amounts of cytoplasm which is pale blue. The cytoplasm is often only a thin rim or crescent which cannot be seen completely encircling the nucleus.

Medium sized lymphocytes occur in blood smears from normal dogs and have more abundant cytoplasm which may completely encircle the nucleus. Medium sized lymphocytes in peripheral blood may approach the size of neutrophils. Occasionally, these lymphocytes will contain several small pink-purple cytoplasmic granules and are referred to as large granular lymphocytes (Fig. 104.8). These granules may be confined to a small cluster near one side of the nucleus. Reactive lymphocytes can be seen in blood smears from dogs responding to an antigenic stimulation. Reactive lymphocytes may be larger and have increased amounts of intensely basophilic cytoplasm (Fig. 104.9). Rarely, they may take on morphology similar to mature plasma cells, with an eccentric nucleus and a perinuclear clear area.

Monocytes are larger than neutrophils (Fig. 104.10). Monocyte nuclei are extremely variable and may be round, multilobated, band-shaped, or S-shaped (Figs. 104.11, 104.12). Those that are band shaped are gener-

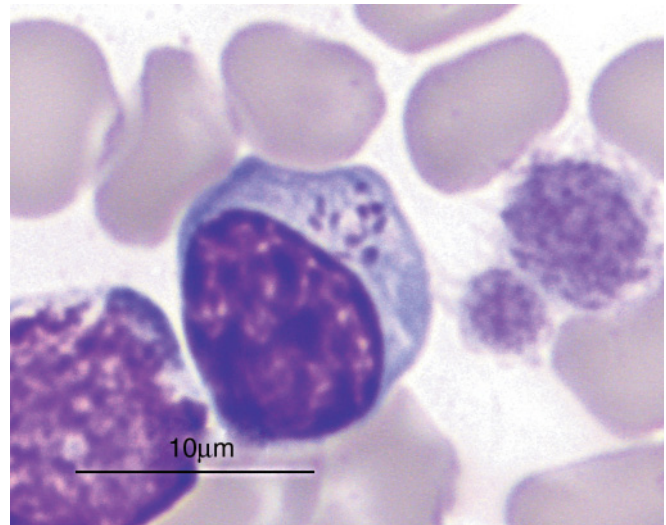


FIGURE 104.8 A large granular lymphocytes with pink-purple granules clustered to one side of the nucleus. Wright-Giemsa stain.

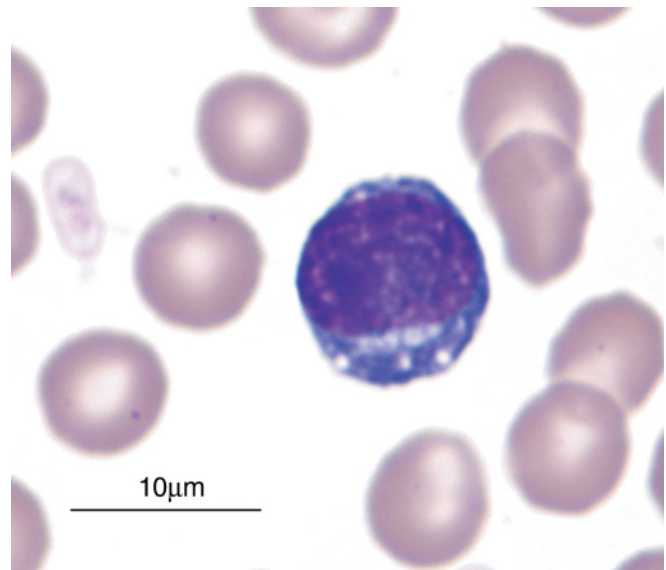


FIGURE 104.9 A reactive lymphocyte with increased volume of intensely basophilic cytoplasm. Wright-Giemsa stain.

ally wider and may have rounded, knob-shaped ends that differentiate them from band neutrophils (Fig. 104.13). The nuclear chromatin is less condensed than that of the mature neutrophil. Their cytoplasm is moderate to abundant and gray-blue to deeply basophilic. Canine monocytes may have variable numbers of clear, distinct-walled cytoplasmic vacuoles. Occasionally, faint, dust-like azurophilic granules can be seen evenly spread throughout the blue cytoplasm.

Eosinophils can usually be found in low numbers on blood smears from healthy dogs. Eosinophils are slightly larger than neutrophils. Their most characteristic feature is the presence of prominent eosinophilic

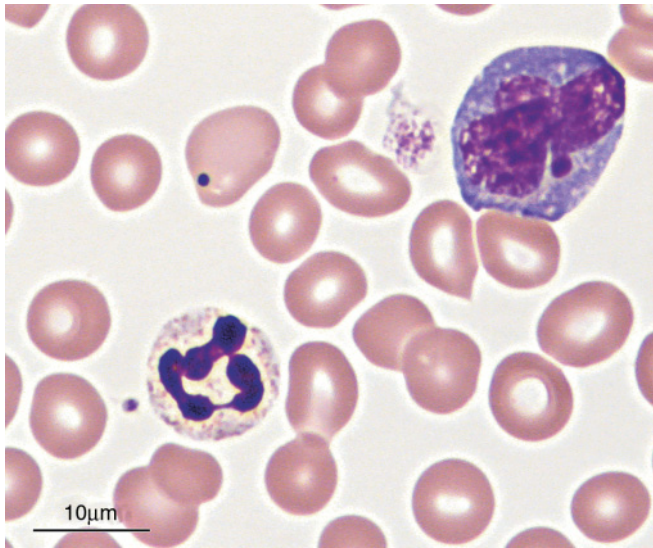


FIGURE 104.10 A neutrophil (lower left) and monocyte (upper right). The monocyte is large and has basophilic cytoplasm. This monocyte does not have cytoplasmic vacuoles, a feature that is variable. Wright-Giemsa stain.

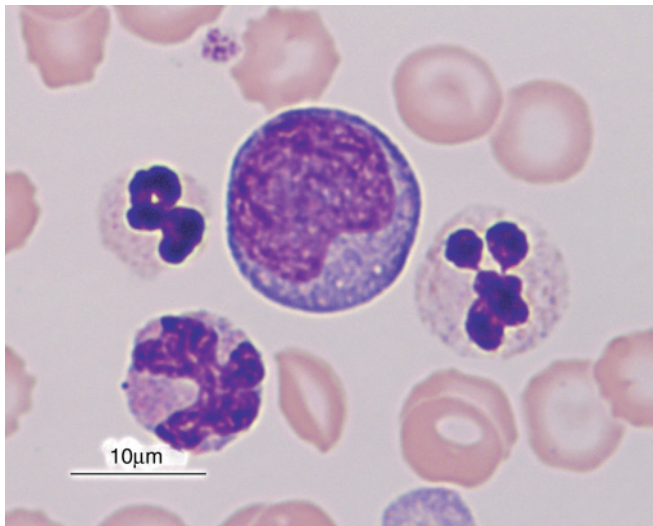


FIGURE 104.11 Monocyte (center) with a rounded, indented nuclear shape. Wright-Giemsa stain.

granules. In dogs, the number and size of these granules is greatly variable (Figs. 104.14 and 104.15). Size of the granules within a single cell may also be variable. Occasionally, an eosinophil may contain only one or two extremely large granules which could be mistaken for an inclusion body or parasite. Cytoplasm between the granules is lightly basophilic and may contain a few clear vacuoles.

Eosinophil nuclei are less lobated than those of neutrophils, usually having only two or three lobes. Eosinophils of adult Greyhounds are unique in that they contain numerous clear vacuoles and often lack

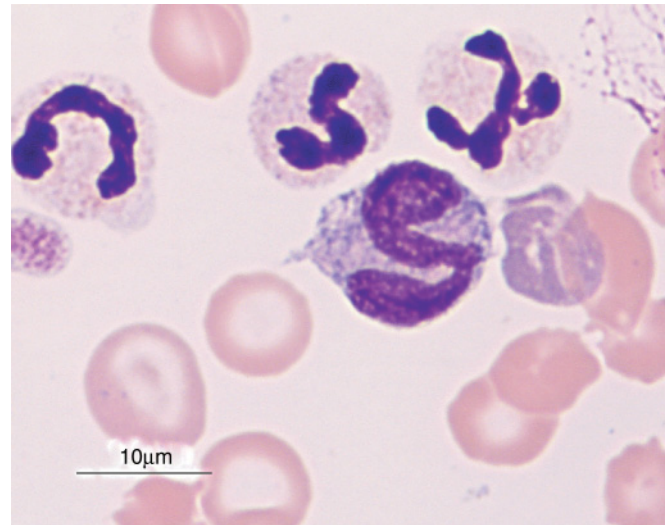


FIGURE 104.12 Monocyte (bottom) with “S” shaped nucleus and three neutrophils. Wright-Giemsa stain.

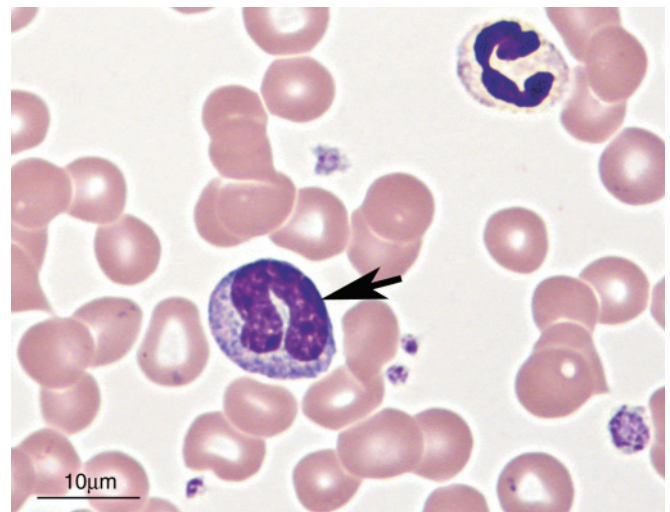


FIGURE 104.13 Monocyte (arrow) with band shaped nucleus. Nucleus is thicker than that of neutrophil (upper right). Wright-Giemsa stain.

distinct granules (Fig. 104.16).¹⁸ Interestingly, eosinophils of Greyhound pups contain granules, although these are a dull slate color rather than orange as in other species.

Basophils are rare in blood smears from normal dogs. Basophils are larger than neutrophils and have a more elongated, convoluted nucleus (Fig. 104.17). Compared to other species, canine basophils contain only scattered, small, purple to pink-purple cytoplasmic granules (Fig. 104.18). Cytoplasm is blue-gray to slightly purple.

Quantitative Parameters

Reference intervals for leukocyte parameters of adult dogs are given in Table 104.1. As noted before, reference

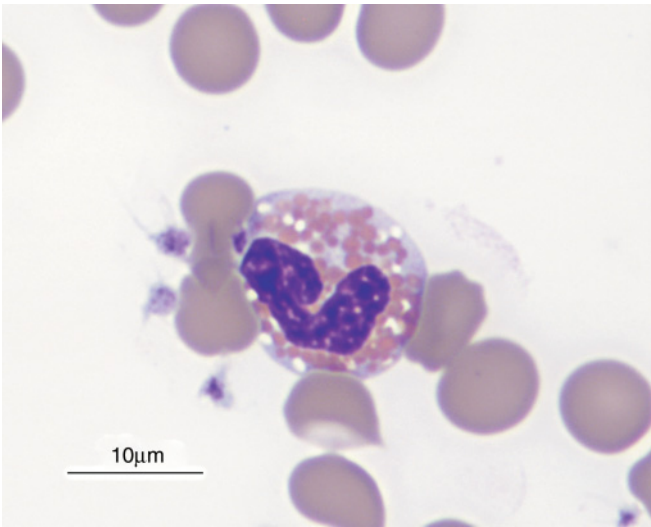


FIGURE 104.14 Canine eosinophil with numerous small granules. Wright-Giemsa stain.

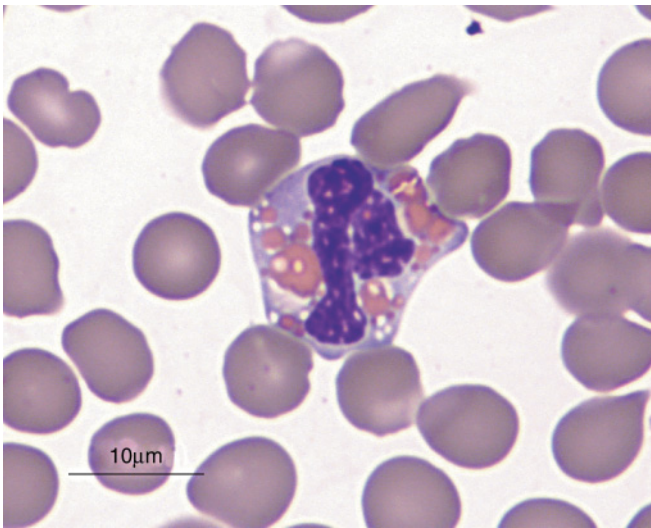


FIGURE 104.15 Canine eosinophil with variably sized granules. Wright-Giemsa stain.

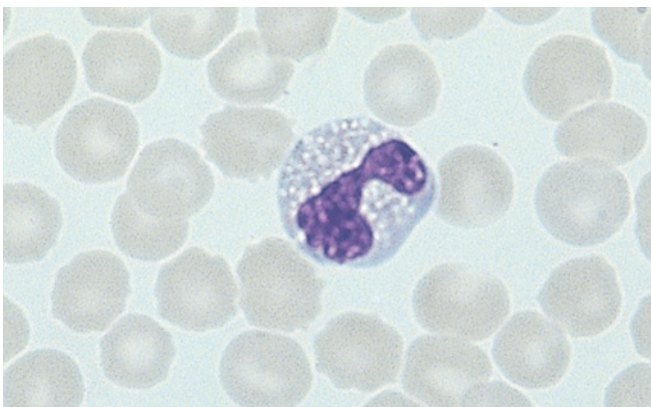


FIGURE 104.16 Vacuolated eosinophil from an adult Greyhound. Wright-Giemsa stain; 100× objective. (Courtesy of Oklahoma State University Clinical Pathology Teaching Files.)

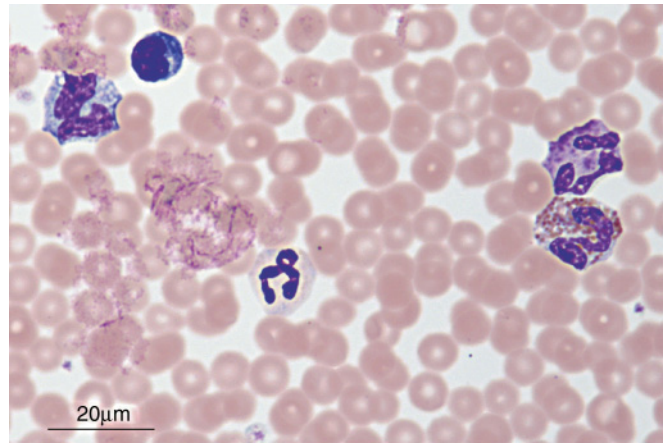


FIGURE 104.17 Monocyte, lymphocyte, neutrophil, basophil (top right) and eosinophil (bottom right). Having all the major leukocyte types in one field allows comparison of size and nuclear shapes. Note the elongated, thin nucleus of the basophil. Wright-Giemsa stain.

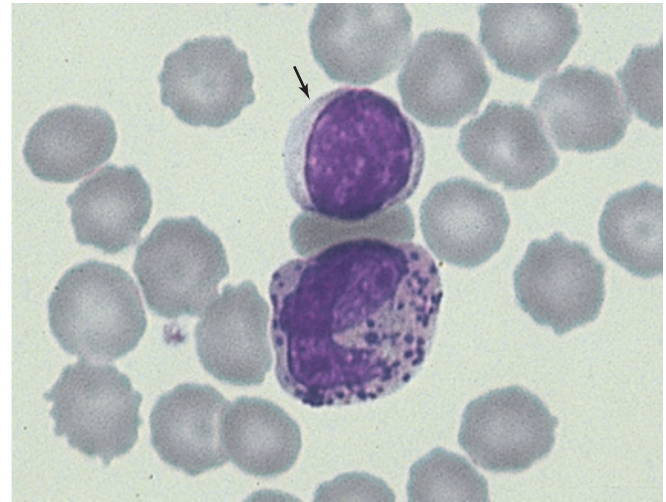


FIGURE 104.18 Canine basophil and medium lymphocyte. Canine basophils have a segmented nucleus and sparse granules. The lymphocyte (arrow) is slightly larger than a small lymphocyte and contains more abundant cytoplasm. Wright-Giemsa stain; 100× objective. (Courtesy of Oklahoma State University Clinical Pathology Teaching Files.)

intervals will be affected by the methodology used and population from which they are derived. This could potentially have a greater effect on leukocyte parameters than on those of other cell lines, since animals not in controlled environments have greater exposure to a variety of antigens. Diurnal and seasonal variations have been reported in leukocyte counts, although the fluctuations are relatively minor and do not exceed the limits of standard reference intervals.^{21,41} In some geographic areas, animals may have higher eosinophil counts in certain seasons due to environmental allergens and parasite load. In addition, short term alterations in leukocyte numbers may occur as part of an epinephrine- or corticosteroid-induced response in

animals that are not conditioned to blood collection (Chapter 48).

Physiologic leukopenia has been reported in healthy adult North American Belgian Tervuren dogs.¹⁴ No difference in leukocyte parameters was found between Belgian Tervuren dogs and other dogs in a more recent study.¹² Study differences in North American Belgian Tervuren and Belgian Tervuren dogs were thought to reflect genetic or environmental factors.¹²

Fluctuations in total white blood cell (WBC) and neutrophil counts have been noted in Beagles during the first 2 months of life; however, with a few exceptions, values generally remained within the reference intervals listed in Table 104.2.³⁹ In one study, newborn animals (0–3 days) had band neutrophil counts that were slightly outside the adult reference interval, indicating a left shift. Occasionally, metamyelocytes were seen during this period without any other evidence of disease. Band neutrophils decreased to within reference limits by 7–10 days of life.³⁹ Lymphocyte counts are significantly higher in young dogs. Dogs less than 6 months of age typically have lymphocyte counts of at least 2,000/ μL , with some as high as 10,000/ μL .³⁹

After maturity, there is little change in hematologic parameters. Studies have found no significant trends in WBC counts of dogs from 1.5 to 14 years of age.^{8,22} During pregnancy, total WBC counts may increase to levels that are slightly above reference intervals ($\sim 19,000/\mu\text{L}$) near parturition. White blood cell counts decrease into the reference interval during lactation, but may not return to baseline values until the pups are weaned.¹

PLATELETS

Morphology

Canine platelets appear as small, oval to round structures on blood smears. Canine platelets are generally one-fourth to one-half the size of erythrocytes, but some platelets are larger than a RBC (Fig. 104.2). They have clear to light gray cytoplasm and numerous small pink to purple granules. The granules may be dispersed throughout the platelet or aggregated into a central cluster. Occasionally, platelets become partially activated and have a spider-like appearance with small cytoplasmic pseudopodia (Fig. 104.19). Alternatively, activated platelets may form small clumps or a large agglutinated mass. Such large masses of platelets generally get pulled out to the feathered edge of a smear.

Quantitative Parameters

Normal parameters for platelets in adult dogs are listed in Table 104.1. Platelet counts between 200,000 and 500,000/ μL are generally considered normal. Low platelet counts have been noted in seemingly healthy racing Greyhounds.^{36,40} The mean platelet count from 60 Greyhounds in one study was $190 \times 10^3/\mu\text{L}$ (90×10^3 – $290 \times 10^3/\mu\text{L}$; mean ± 2 SD). No dog had increased platelet surface associated immunoglobulin.³⁶

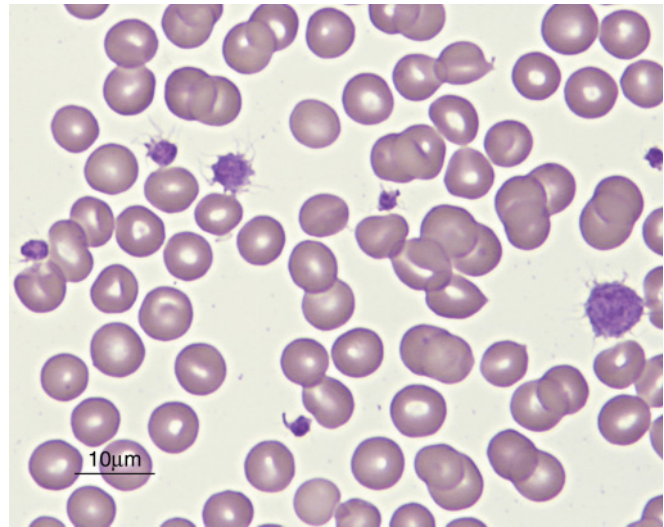


FIGURE 104.19 Canine platelets with long, filamentous cytoplasmic projections. Wright-Giemsa stain.

Asymptomatic thrombocytopenia with enlarged platelets (macrothrombocytopenia) is common in Cavalier King Charles spaniels.^{3,32} The condition is associated with a mutation in the $\beta 1$ -tubulin gene.⁶ The median platelet count from 105 Cavalier King Charles spaniels in one study was $92 \times 10^3/\mu\text{L}$ (25 – $394 \times 10^3/\mu\text{L}$) with 56% of the dogs having platelet counts less than $100 \times 10^3/\mu\text{L}$.³² Analyzers that assess “plateletcrit” rather than impedance-based or optical-based particle counting have shown the total platelet mass in affected animals to be normal.⁴²

Mean platelet volumes (MPV) from colony raised Beagles has been reported to be $8.5 (\pm 0.53)$ fL.⁴³ Reference intervals vary significantly between analyzers (see Table 104.1).

BONE MARROW

Morphology

Marrow samples in the dog are commonly collected from the trochanteric fossa of the femur, proximal humerus and the iliac crest. The morphology of canine hematopoietic cells found in bone marrow aspirates has been reviewed.¹⁶ Morphologic assessment consists of an evaluation of the major hematopoietic cells lines (i.e. erythroid, granulocytic and megakaryocytic) as well as other cells normally found in the marrow (i.e. lymphocytes, plasma cells and macrophages).

Erythroid Series

Stages of nucleated erythroid precursors recognizable in marrow aspirates are rubriblasts, prorubricytes, rubricytes, and metarubricytes. With progressive maturation, the nucleated cells become smaller, undergo nuclear condensation, and cytoplasm color changes from deeply basophilic to red-orange (Figs. 104.20 and 104.21).

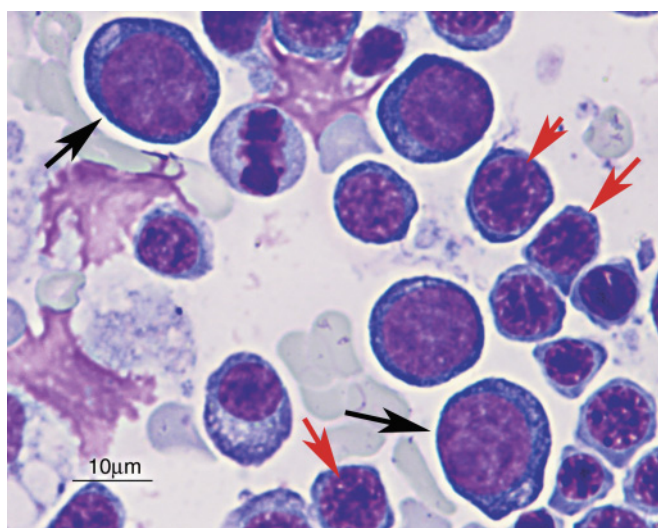


FIGURE 104.20 Canine bone marrow showing developing erythroid cells. Rubriblasts (black arrows) have nucleoli, round nuclei and deeply basophilic cytoplasm. Developing rubricytes (red arrows) are small with coarse chromatin. Diff-Quick stain.

Rubriblasts are present in relatively low numbers. They are a large cell with a high N:C ratio. The nucleus is nearly perfectly round with fine chromatin and one or more visible nucleoli (Fig. 104.20). The cell has moderate amounts of deeply basophilic cytoplasm. Prorubricytes are slightly smaller, lack visible nucleoli and have somewhat coarser chromatin. The N:C ratio is slightly lower and cytoplasm remains intensely basophilic.

The next stage in maturation is the rubricyte. Rubricytes are decidedly smaller with a lower N:C ratio. The nucleus is smaller, has extremely coarse chromatin and nucleoli are not visible (Figs. 104.20 and 104.21). The cytoplasm undergoes a progressive color change from deeply basophilic to polychromatophilic (blue-orange) to red-orange representing progressive production of Hgb. Rubricytes are sometimes separated into three types (basophilic, polychromatophilic and orthochromic) based predominantly on change in cytoplasm color. Metarubricytes are the smallest of the nucleated erythroid precursors and are characterized by a pyknotic nucleus that lacks a discernible chromatin pattern (Fig. 104.21). Progressive maturation results in polychromatophilic erythrocytes, non-nucleated cells similar to those seen in low numbers in the peripheral blood.

Granulocytic Series

The granulocytic series goes through a similar progression of relatively low numbers of early precursors to more numerous maturing cells (Fig. 104.22). The recognizable stages of granulocytic maturation in the marrow are myeloblasts, promyelocytes (also called “progranulocytes”), myelocytes, metamyelocytes, bands and mature granulocytes.

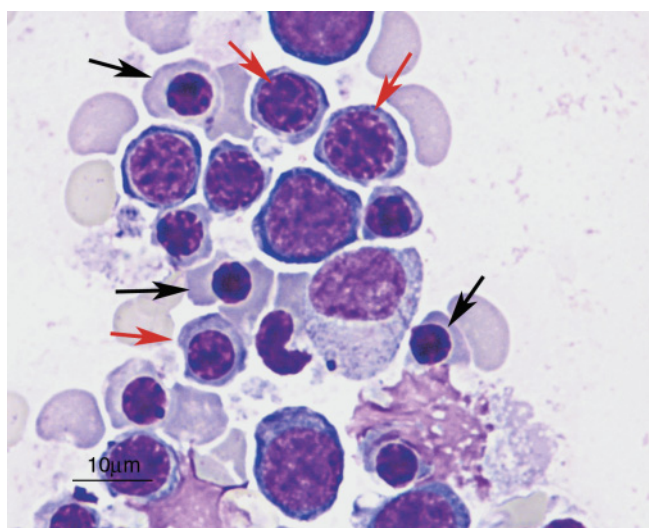


FIGURE 104.21 Canine bone marrow. Developing rubricytes (red arrows) have coarse chromatin. Metarubricytes (black arrows) have condensed pyknotic nuclei. Diff-Quick stain.

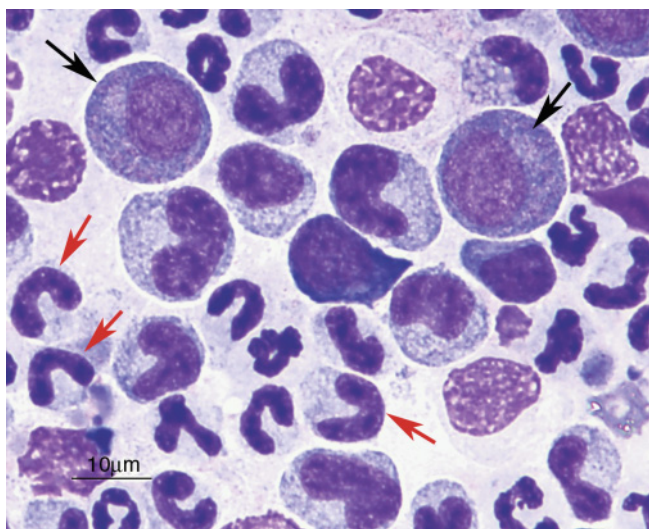


FIGURE 104.22 Canine bone marrow. Developing myeloid cells are shown. Promyelocytes (black arrows) are recognized by the presence of pink-purple primary granules. Bands (red arrows) are more numerous. A few large metamyelocytes with indented, but not band shaped, nuclei are also present. Diff-Quick stain.

Myeloblasts are large cells with a high N:C ratio, fine chromatin and one or more visible nucleoli. Compared to rubriblasts, the myeloblast has a more irregularly shaped nucleus and lighter staining cytoplasm. The next stage in granulocytic development is the promyelocyte. Often, these cells are slightly larger than myeloblasts but have a lower N:C ratio due to cytoplasmic enlargement. Nuclear chromatin is somewhat coarser. Nucleoli may or may not be present. The main feature identifying the promyelocyte is the presence of numerous fine, azurophilic, granules termed primary granules, in the cytoplasm (Fig. 104.22). These granules help

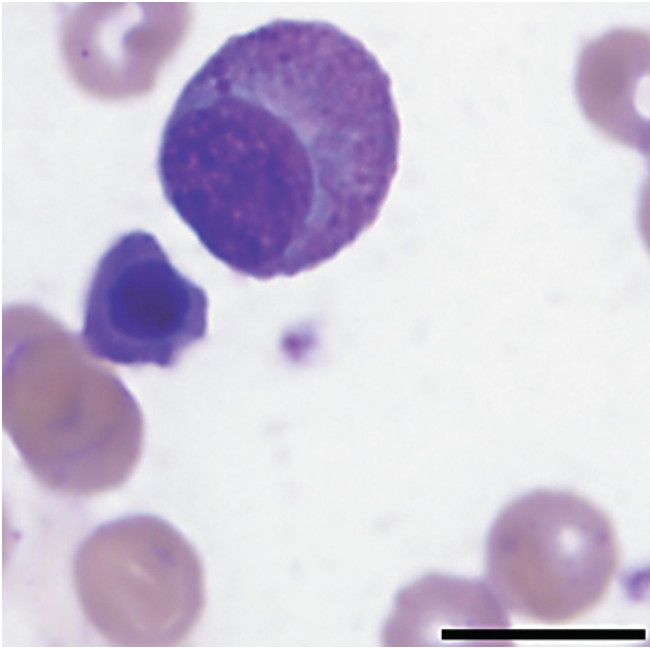


FIGURE 104.23 Canine bone marrow. An eosinophilic myelocyte (upper right) is identifiable due to the presence of developing secondary, eosinophilic granules. Wright-Giemsa stain.

identify the cell as one of granulocytic origin. Myelocytes, smaller than promyelocytes, are characterized by loss of visible primary granules, round to oval nuclei with dense chromatin and no visible nucleoli. Cytoplasm becomes progressively lighter to clear and secondary granules may be present identifying the cells as eosinophils or basophils (Fig. 104.23). Secondary granules in neutrophilic cells are generally indistinct.

Metamyelocytes are the next stage in maturation and are characterized by the beginnings of indentation of the nucleus. The nucleus often has a kidney bean shape. If the indentation is $>25\%$ of the thickness of the nucleus, then the cell is considered a metamyelocyte while those with less of an indentation are considered myelocytes. Cytoplasm is clear, with the presence of prominent secondary granules in the eosinophilic and basophilic precursors. Progressive indentation of the nucleus gives rise to the “band” cell which has a rod shaped or curved band shaped nucleus with parallel sides and no indentations/constrictions. The final stage is the mature granulocyte characterized by nuclear constrictions giving rise to the classic polylobated appearance. If the nucleus of the cell has any constrictions that are less than half the diameter of the remainder of the nucleus, the cell is characterized as a mature granulocyte. Cytoplasm remains clear in neutrophilic precursors and has well formed granules in the eosinophilic and basophilic cells.

Megakaryocytic Series

Cells of the megakaryocytic series are classified as megakaryoblasts, promegakaryocytes and megakaryocytes. These cells are typically identified on low magnification

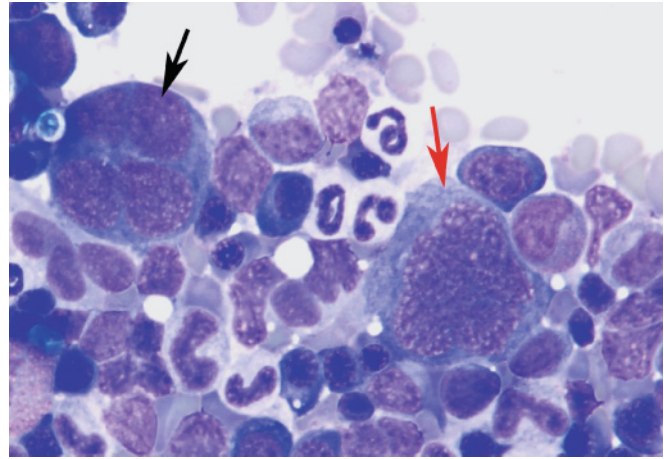


FIGURE 104.24 Canine bone marrow. A promegakaryocyte (black arrow) is recognizable as a large cell with four visible nuclei. An early megakaryocyte (red arrow) has a large, convoluted nuclear mass from multiple nuclear reduplications but retains deeply basophilic cytoplasm. Diff-Quick stain.

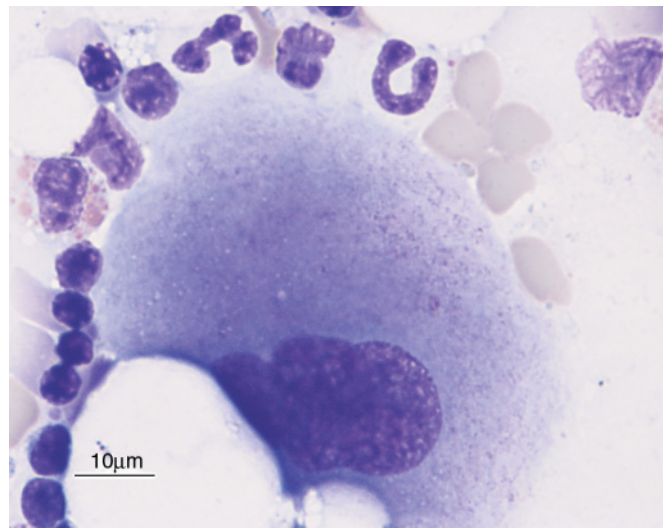


FIGURE 104.25 Canine bone marrow. A mature megakaryocyte is shown with abundant cytoplasm that has pink-purple granules similar to those seen in peripheral blood platelets. (Diff-Quick stain)

as most are substantially larger than the other hematopoietic precursors. Megakaryoblasts are present in low numbers. They have a high N:C ratio, single nucleus and deeply basophilic cytoplasm. It is difficult to reliably differentiate them from blasts of other cell lines. Promegakaryocytes are more easily identified. Endomitotic reduplication of the nucleus results in a large cell with 2–4 nuclei (Fig. 104.24). These may appear to be separate or may be seen to be connected by strands of nuclear material. They have more abundant, deeply basophilic cytoplasm than the megakaryoblast. Further maturation with progressive reduplication of the nucleus gives rise to variably sized megakaryocytes that have an enlarging polylobated nuclear mass (Figs. 104.24 and

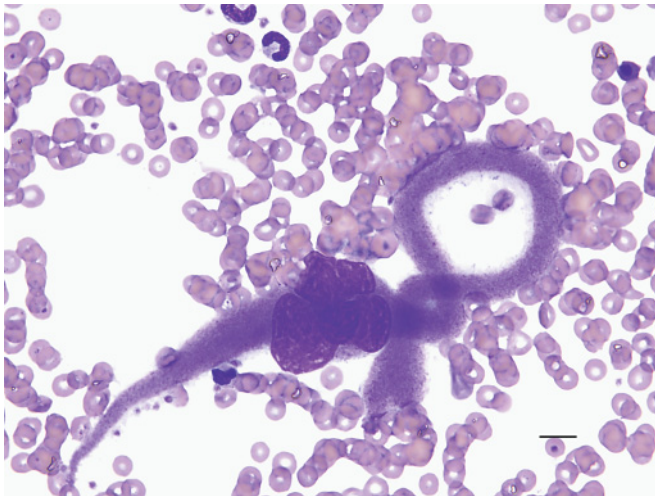


FIGURE 104.26 Canine bone marrow. A mature megakaryocyte is seen with elongated strands of mature, granular cytoplasm. These cytoplasmic extensions would have shed platelets into circulation by fragmentation. Wright-Giemsa stain.

104.25). As megakaryocytes mature, the cytoplasm becomes more abundant and changes from deeply basophilic to eosinophilic and granular, having an appearance similar to platelets (Figs. 104.24, 104.25 and 104.26).

Cells of the monocytic series comprise only a small percentage of the cells in normal marrow. Mature monocytes resemble those in peripheral blood. Cells of the earlier stages, monoblasts and promonocytes, are difficult to clearly separate from granulocytic precursors. Their nuclear shape is more irregular and they lack the primary granules seen in promyelocytes.

Mature lymphocytes may be present in moderate numbers, and low numbers of plasma cells and mature macrophages can be seen admixed amongst the hematopoietic precursors.

Quantitative Parameters

The relative proportion of hematopoietic cells found in samples of bone marrow from normal dogs is given in Table 104.3.^{25,28} The M:E ratio of canine marrow is generally reported as being between 1.0:1 and 2.0:1. In one study, normal males had a significantly higher M:E ratio than females, some ranging as high as 2.9:1.³³ Comparison of marrow samples collected from various anatomical sites has shown that the percentages of various cellular elements are fairly uniform.^{33,35} Also, repeated sampling of bone marrow from the same anatomic location seems to have little effect on differential cell counts.^{17,29} Neonatal dogs have a significantly increased percentage of lymphocytes in bone marrow samples compared to adults.¹⁰ In one study, mean lymphocyte percentages peaked at 54.7% by 21 days of age decreasing to 17.6% by 56 days. Bone marrow samples from normal adult dogs generally have less than 10% lymphocytes.

TABLE 104.3 Differential Cell Counts from Bone Marrow Samples of Normal Dogs

Cell Type	Mean \pm SD ^a (%)	Mean \pm SD ^b (%)
Myeloid series		
Myeloblasts	0.9 \pm 0.2	0.11 \pm 0.14
Progranulocytes	2.1 \pm 0.4	2.27 \pm 0.99
Neutrophilic myelocytes	6.3 \pm 1.0	2.76 \pm 0.98
Neutrophilic metamyelocytes	7.9 \pm 2.1	5.80 \pm 1.71
Neutrophilic bands	11.3 \pm 2.2	26.1 \pm 5.82
Neutrophils	23.5 \pm 1.3	3.32 \pm 1.85
Eosinophilic myelocytes	0.6 \pm 0.2	0.57 \pm 0.47
Eosinophilic metamyelocytes	0.7 \pm 0.3	0.85 \pm 0.62
Eosinophilic bands	1.2 \pm 0.4	1.30 \pm 0.97
Eosinophils	0.8 \pm 0.5	0.20 \pm 0.22
Basophils	0.02 \pm 0.04	0.00 \pm 0.01
Total myeloid series	55.32	43.7 \pm 8.68
Erythroid series		
Rubriblasts, prorubricytes	6.5 \pm 0.5	4.4 \pm 1.23
Rubricytes, metarubricytes	27.6 \pm 4.4	40.59
Total erythroid series	34.1	45.0 \pm 9.81
Myleoid:erythroid ratio	1.7 \pm 0.4	1.8 \pm 0.61
Lymphocytes	8.2 \pm 2.7	6.39 \pm 3.75
Plasma cells	0.7 \pm 0.3	2.98 \pm 1.65
Mitotic figures	1.4 \pm 0.3	1.14 \pm 0.53
Macrophages	0.4 \pm 0.2	0.22 \pm 0.24
Megakaryopoietic cells	—	0.23 \pm 0.20

^aAdapted from Melveger BA, Earl FL, Van Loon ED. Sternal bone marrow biopsy in the dog. *Lab Anim Care* 1969;19:866–868.

^bAdapted from Mischke R, Busse L. Reference values for the bone marrow aspirates in adult dogs. *J Vet Med* 2002;49:499–502.

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Normal Hematology of the Cat

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Hct, hematocrit; Hgb, hemoglobin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; M:E ratio, myeloid:erythroid ratio; MPV, mean platelet volume; N:C ratio, nuclear:cytoplasmic ratio; RBC, red blood cell; RNA, ribonucleic acid; WBC, white blood cell.

ERYTHROCYTES

Morphology

The feline erythrocyte is a biconcave disc, 5.5–6.3 μm in diameter.¹³ They typically lack discernible pale centers on blood smears and exhibit mild anisocytosis (Fig. 105.1). When stained with Romanowsky stains, such as Wright's stain, rare polychromatophilic (gray-blue staining) cells may be seen, but these comprise less than 0.4% of erythrocytes (Fig. 105.2).⁴ These polychromatophilic cells are erythrocytes recently released from the bone marrow and when stained with a supravital stain, such as new methylene blue, are aggregate reticulocytes.¹ Feline reticulocytes are classified as aggregates and punctates (Fig. 105.3). Aggregates are larger than mature erythrocytes with varying amounts of residual ribonucleic acid (RNA) and organelles (polyribosomes and mitochondria). Aggregate reticulocytes are the youngest anucleate erythrocytes. Punctate reticulocytes may be similar in size to mature erythrocytes and contain less RNA. Feline reticulocytes appear to have prolonged maturation times and punctate reticulocytes can be identified in new methylene blue stained preparations 2–3 weeks after blood loss.¹⁴ Supravital stains, such as cresyl blue or new methylene blue, penetrate the cell

membrane and cause the variable amounts of RNA to clump (aggregate reticulocytes) or diffusely precipitate (punctate reticulocytes) depending on their age.

Moderate rouleaux can be observed on blood smears from normal cats, and 0–1% of red blood cells (RBCs) can contain Howell-Jolly bodies (Fig. 105.1). Low numbers of Heinz bodies (less than 5% of RBCs) can be found on blood smears of some healthy, nonanemic cats¹¹ (Fig. 105.4).

Two blood groups have been reported in cats and are designated the AB system and the recently recognized Mik red cell antigen reported in 2007 in four cats.²³ In one study of geographic variation of blood groups, the prevalence of type A (98.2%) was much higher in domestic shorthair/domestic longhair (DSH/DLH) populations than type B (1.7%) and only rarely type AB (0.1%) was encountered.⁶ In the same study, the prevalence of blood type B increased in purebred populations. The frequency of blood type B blood in Abyssinian was 13.5%, Persian 9.6%, and Devon Rex cats 49.7%.⁶ Sera of either type A or type B cats can contain naturally occurring isoagglutinins to the opposite blood type.⁸ Blood type AB cats do not contain anti-A or -B isoagglutinins. The anti-A isoantibodies in type B cats are highly agglutinating and hemolytic to both blood type A and blood type AB.⁸

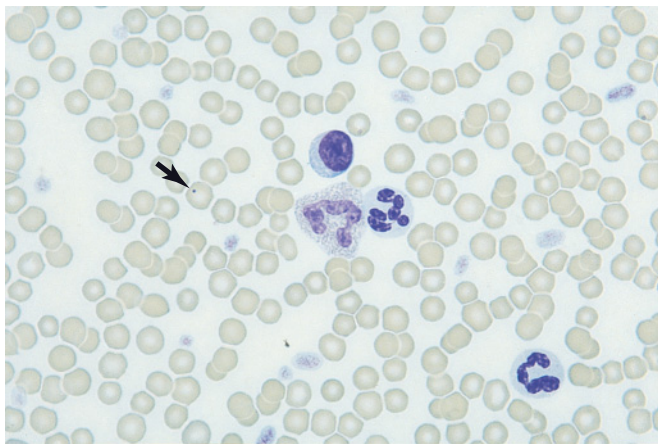


FIGURE 105.1 Feline RBCs exhibit moderate anisocytosis. Pale centers are frequently not readily apparent. A Howell-Jolly body (arrow), two segmented neutrophils, a lymphocyte, an eosinophil, and both round and elongated platelets are present. Wright-Giemsa stain; 100× objective.

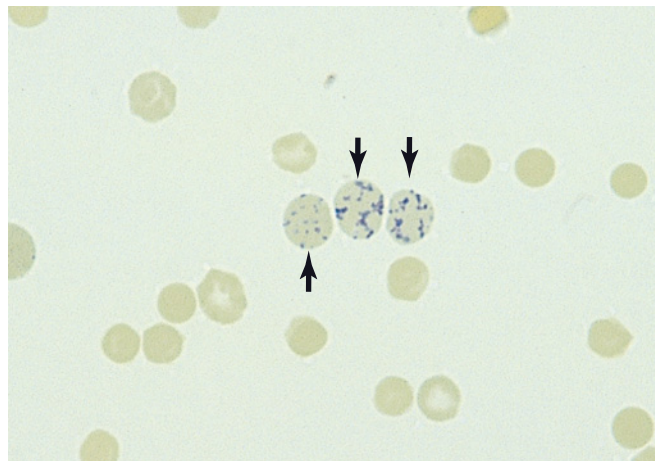


FIGURE 105.3 Two feline aggregate reticulocytes with markedly aggregated reticulation (top arrows) and one punctate reticulocyte with slight focal reticulation (bottom arrow) are present. New methylene blue stain; 100× objective.

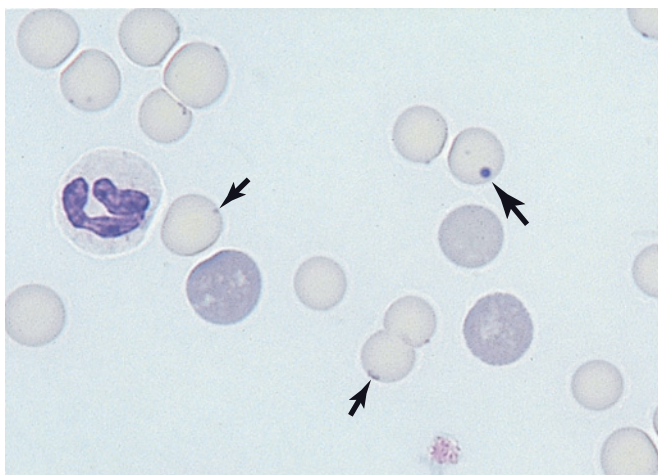


FIGURE 105.2 Macrocytic gray-blue polychromatophilic RBCs and normal feline RBCs are present. A single Howell-Jolly body (large arrow) is seen in contrast to many rod-shaped, peripherally located *Mycoplasma haemofelis* organisms (small arrows). A few platelets and a segmented neutrophil are also seen. Wright-Giemsa stain; 100× objective.

Quantitative Parameters

Hemoglobin, Hematocrit, and Red Blood Cell Count

The hemoglobin (Hgb), hematocrit (Hct) and RBC count are all interrelated measurements of erythrocyte mass; in the absence of significant alterations in erythrocyte size and Hgb concentration, changes in these parameters tend to parallel each other. Hematocrit is the most frequently used parameter by practitioners to evaluate RBC mass.

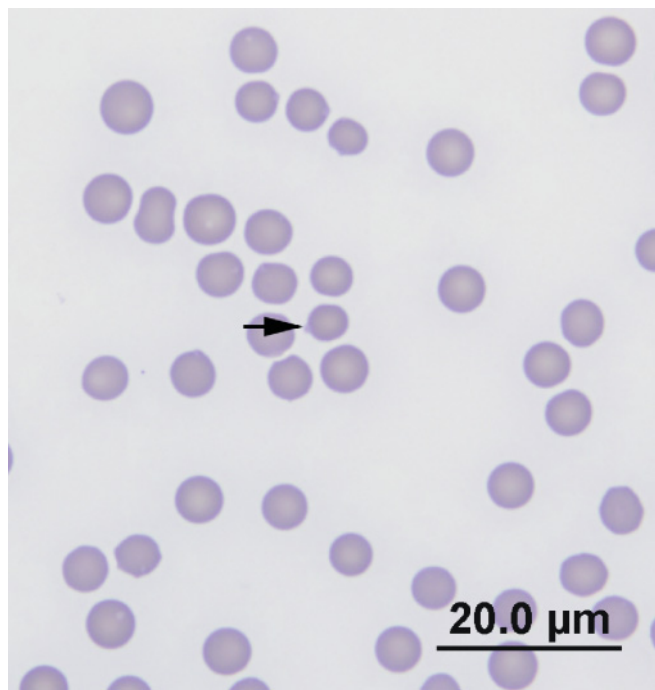


FIGURE 105.4 Feline RBCs with Heinz body (arrow). Low numbers of Heinz bodies may be seen on a blood film from normal cats. Aqueous Romanowsky stain.

Several sets of reference normal values are available for kittens. The normal hematologic values for kittens are presented in Table 105.1. The agreement among reference values for adult cats is better, particularly for the means. The ranges for WBC and absolute neutrophil counts vary the most; this is likely caused by varying degrees of physiologic leukocytosis present in various sampling groups of normal cats. The normal

TABLE 105.1 Blood Values of Normal Juvenile Cats to 18 Weeks of Age^a

	Age (weeks)							
	0-2	2-4	4-6	6-8	8-9	12-13	16-17	18 ^b
PCV (%)	35.3 ± 1.7	26.5 ± 0.8	27.1 ± 0.8	29.8 ± 1.3	33.3 ± 0.7	33.1 ± 1.6	34.9 ± 1.1	34.6 ± 3.6
Hgb (g/dL)	12.1 ± 0.6	8.7 ± 0.2	8.6 ± 0.3	9.1 ± 0.3	9.8 ± 0.2	10.1 ± 0.3	11 ± 0.4	10.9 ± 1.5
RBC ($\times 10^6/\mu\text{L}$)	5.23 ± 0.24	4.67 ± 0.1	5.89 ± 0.23	6.57 ± 0.26	6.95 ± 0.09	7.43 ± 0.23	8.14 ± 0.27	7.3 ± 0.8
MCV (fL)	67.4 ± 1.9	53.9 ± 1.2	45.6 ± 1.3	45.6 ± 1.0	47.8 ± 0.9	44.5 ± 1.8	43.1 ± 1.5	48.1 ± 5.1
MCH (pg)	23.0 ± 0.6	18.8 ± 0.8	14.8 ± 0.6	13.9 ± 0.3	14.1 ± 0.2	13.7 ± 0.4	13.5 ± 0.4	15.1 ± 2.1
MCHC (g/dL)	34.5 ± 0.8	33.0 ± 0.5	31.9 ± 0.6	30.9 ± 0.5	29.5 ± 0.4	31.3 ± 0.9	31.6 ± 0.8	31.4 ± 3.2
WBC ($\times 10^3/\mu\text{L}$)	9.67 ± 0.57	15.31 ± 1.21	17.45 ± 1.37	18.07 ± 1.94	23.68 ± 1.89	23.20 ± 3.36	19.70 ± 1.12	11.9 ± 7.1
Neutrophils	5.96 ± 0.68	6.92 ± 0.77	9.57 ± 1.65	6.75 ± 1.03	11.0 ± 1.41	11.0 ± 1.77	9.74 ± 0.92	NR
Lymphocytes	3.73 ± 0.52	6.56 ± 0.59	6.41 ± 0.77	9.59 ± 1.57	10.17 ± 1.71	10.46 ± 2.61	8.78 ± 1.06	NR
Monocytes	0.01 ± 0.01	0.02 ± 0.02	0	0.01 ± 0.01	0.11 ± 0.06	0	0.02 ± 0.02	NR
Eosinophils	0.96 ± 0.43	1.40 ± 0.16	1.47 ± 0.25	1.08 ± 0.2	2.28 ± 0.31	1.55 ± 0.35	1.00 ± 0.19	NR
Basophils	0.02 ± 0.01	0	0	0.02 ± 0.02	0	0.03 ± 0.03	0	NR

^aAdapted from reference 16.^bAdapted from reference 5.^cNR, not reported.**TABLE 105.2 Hematologic Reference Ranges for Normal Cats**

	Jain 1986 ^a	Moritz et al. 2004 ^b
Erythrogram		
RBC ($\times 10^6/\mu\text{L}$)	5.0–10.0	5.92–11.16
Hemoglobin (g/dL)	8.0–15.0	8.17–15.26
PCV (%)	24.0–45.0	24.0–46.0
MCV (fL)	39.0–55.0	36.96–54.98
MCHC (%)	31.0–35.0	26.24–35.91
Reticulocytes		
Aggregate (%)	0–0.4	0.13–0.40 ^c
Punctate (%)	1.4–10.8	
Platelet count (/μL)	300,000–800,000	200,670–377,000
MPV (fL)	12–17 ^d	12.47–17.60
Leukogram		
Leukocytes (/μL)	5,500–19,500	10,570–14,390
Band neutrophils	0–300	
Segmented neutrophils	2,500–12,500	6,100–9,480
Lymphocytes	1,500–7,000	2,410–3,990
Monocytes	0–850	290–470
Eosinophils	0–1,500	200–610
Basophils	Rare	10–30
Other data		
RBC diameter (μm)	5.5–6.3	
Erythrocyte lifespan (days)	66–78	

^aReference 12.^bReference 17.^cReference 4.^dReference 25.

hematologic values for adult cats are presented in Table 105.2.

Hematologic reference intervals are available from both research colonies and randomly sampled healthy cats.^{2,5,7,12} These reference intervals show satisfactory agreement for the means of most values with the widest variation occurring in the white blood cell (WBC) count.

Age, sex, nutritional status, and husbandry as well as pregnancy, anesthesia, or pharmacologic treatment can affect the measured hematologic values. The effects of most of these factors on hematologic values are not peculiar to the cat, but are common for all domestic species. Queens can exhibit a mild normocytic, normochromic anemia during the last third of pregnancy; this resolves by 1 week post-parturition.³

Mean Corpuscular Volume, Mean Corpuscular Hemoglobin, and Mean Corpuscular Hemoglobin Concentration

The indices mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) characterize the size and degree of hemoglobinization of the erythrocyte populations.

At birth, feline fetal erythrocytes are larger than those of adults. The MCV is approximately 67 fL at birth and decreases as the cat matures. The nadirs of means for RBC and Hct can be as low as $4.8 \times 10^6/\mu\text{L}$ and 26%, respectively.² Fetal RBCs are replaced by 1–4 months of age, resulting in the values for RBC, Hgb, Hct, and MCV reaching those for the adult during this time.

LEUKOCYTES

Normal Morphology

The WBC and differential counts in cats are more variable than those for dogs. This may be caused in part by a higher percentage of leukocytes in the marginated pool, estimated to be 70%, than in other domestic species.¹⁸ Increased blood flow caused by anxiety shifts leukocytes from the marginated pool to the circulating pool, resulting in higher and more variable WBC and differential counts (physiologic leukocytosis). At birth, the WBC and differential counts are typically within the adult reference interval. For kittens between 3 and 4

months of age, the WBC count may increase to $23 \times 10^3/\mu\text{L}$ and be composed of 50% segmented neutrophils and 50% lymphocytes.^{2,5} The WBC count declines to adult normal range for cats at 5–6 months of age.

The morphology of feline neutrophils is similar to that of other domestic species with segmented nuclei with condensed chromatin and clear to slightly eosinophilic staining cytoplasm (Fig. 105.5). Bar bodies or “drumstick” protrusions of the nucleus can be present in 4–11% of neutrophils of female cats (Fig. 105.6).¹⁵

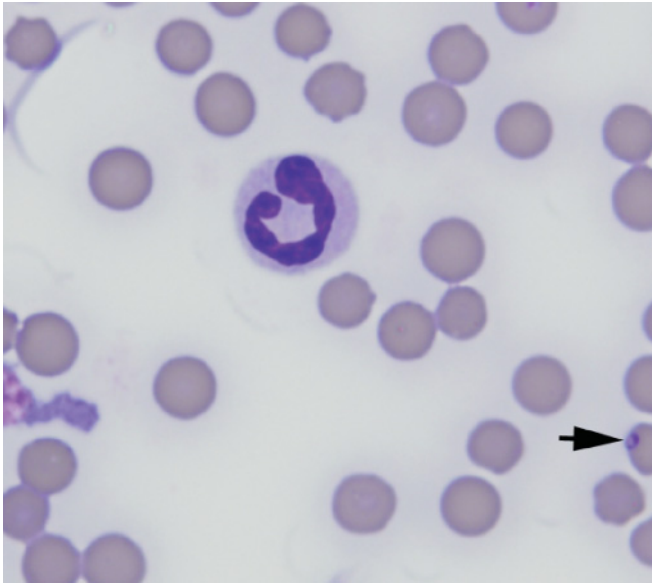


FIGURE 105.5 Feline neutrophil with segmented nuclei with condensed chromatin and an intraerythrocytic *Cytauxzoonan felis* organism (arrow). Aqueous Romanowsky stain.

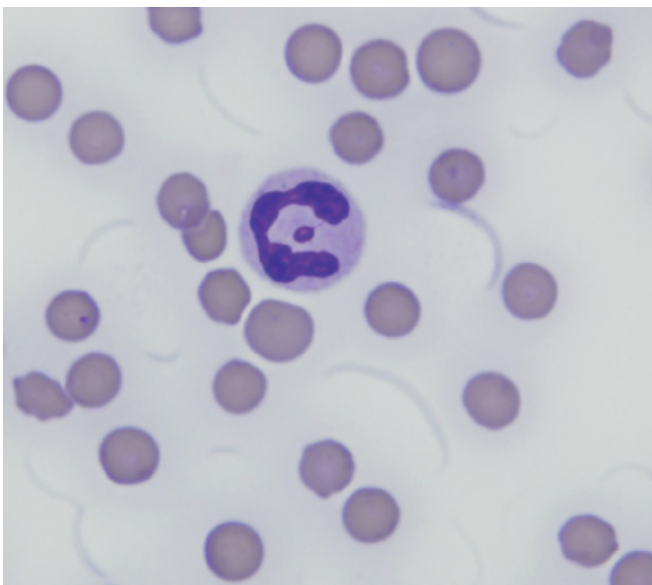


FIGURE 105.6 Feline neutrophil with bar body or “drumstick” protrusion attached to the nucleus with a thin filamentous strand. Aqueous Romanowsky stain.

A hereditary anomaly of granulation in neutrophils of Birman cats has been described.¹⁰ The neutrophils have fine eosinophilic granules in the cytoplasm and do not have any functional abnormalities. Pelger-Huët anomaly has been reported in cats.¹⁴

Eosinophils typically are slightly larger than neutrophils and have segmented nuclei, which are often bilobated with condensed chromatin, and the cytoplasm has abundant, small, uniform-sized, rod-shaped, pale orange staining granules (Fig. 105.7).

Basophils are similar in size to eosinophils and have segmented nuclei with condensed chromatin and abundant, small, uniform-sized, round-shaped, pale lavender to pink staining cytoplasmic granules (Fig. 105.8).

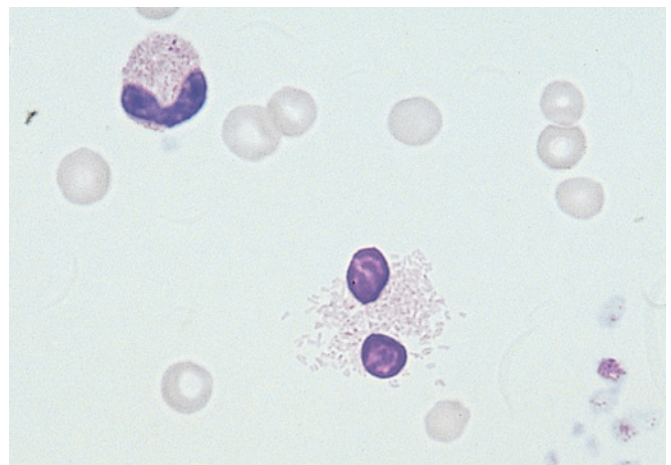


FIGURE 105.7 A ruptured eosinophil demonstrates the rod-shaped pale orange cytoplasmic granules of feline eosinophils. An intact eosinophil is also present. Wright-Giemsa stain.

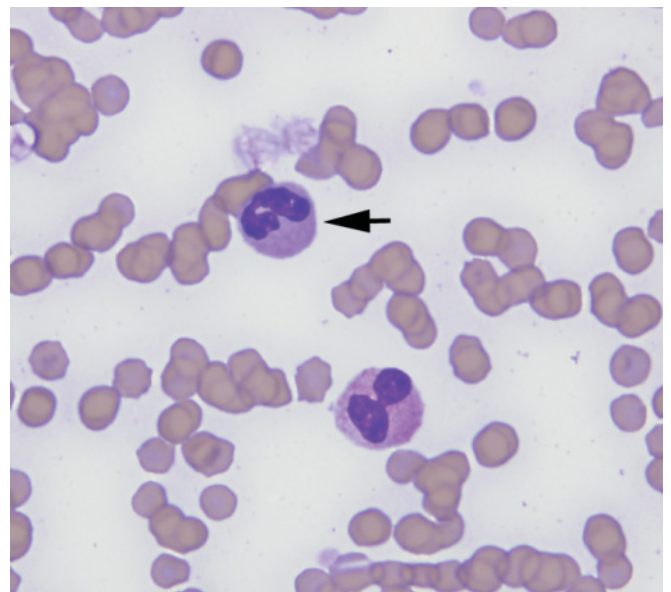


FIGURE 105.8 A feline basophil (arrow) with lavender, round, intracytoplasmic granules and a feline eosinophil. Platelets and rouleaux are also present. Aqueous Romanowsky stain.

A few less-mature dark purple granules may be observed occasionally in feline basophils.

Lymphocytes are smaller than neutrophils, have round to cleaved nuclei, condensed chromatin that appears smudged and lacks discernible nucleoli, and scant basophilic cytoplasm that often appears to extend only one-third of the way around the nucleus (Fig. 105.9). Some lymphocytes can have a few small azurophilic cytoplasmic granules (Fig. 105.10).

Monocytes are larger than neutrophils; have rounded, strap-, bi-, or trilobated or amoeboid-shaped nuclei with reticulated chromatin pattern; and abundant lightly basophilic to gray cytoplasm with a few to several clear variable-sized cytoplasmic vacuoles (Fig. 105.11).

PLATELETS

Feline platelets have morphology similar to other domestic animals except platelets are often macrocytic and occasional elongated forms may be encountered on blood smears (Fig. 105.12). Feline platelet size often overlaps with the size of erythrocytes and they are also prone to clumping after blood collection. The combination of macrocytic platelets and the propensity of feline

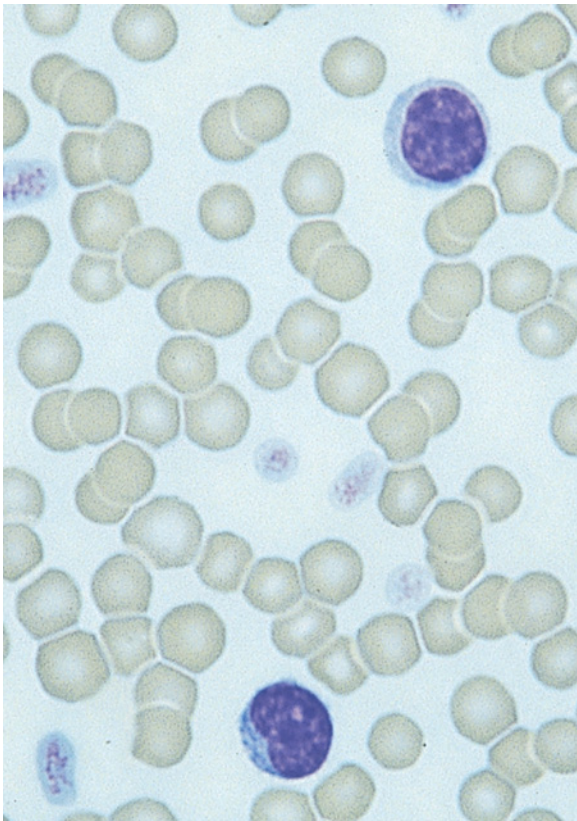


FIGURE 105.9 Two lymphocytes and round and elongated platelets are present. Wright-Giemsa stain; 100× objective. (Courtesy of Oklahoma State University, College of Veterinary Medicine Teaching Files.)



FIGURE 105.10 A lymphocyte containing small azurophilic intracytoplasmic granules (arrow). Wright-Giemsa stain; 100× objective. (Courtesy of Oklahoma State University, College of Veterinary Medicine Teaching Files.)

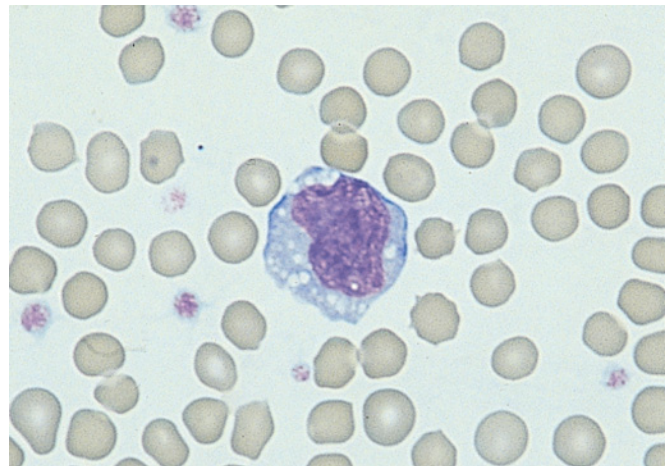


FIGURE 105.11 A monocyte with numerous cytoplasmic vacuoles. Wright-Giemsa stain; 100× objective. (Courtesy of Oklahoma State University, College of Veterinary Medicine Teaching Files.)

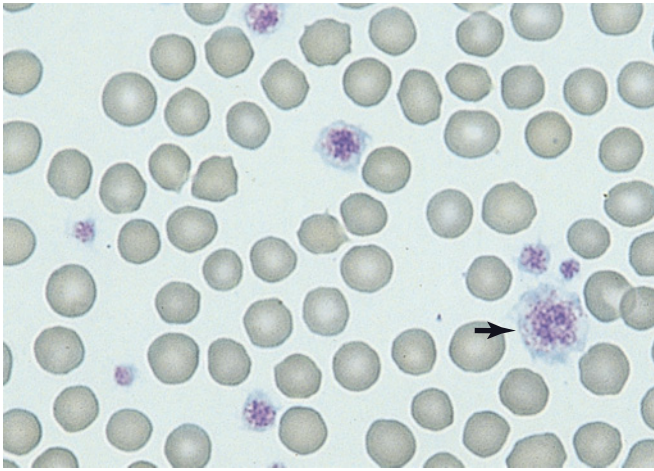


FIGURE 105.12 A large platelet (arrow) and normal-sized platelets. Wright-Giemsa stain; 100× objective. (Courtesy of Oklahoma State University, College of Veterinary Medicine Teaching Files.)

platelets to clump often cause falsely decreased automated platelet counts. The examination of a peripheral blood film can reliably estimate platelet counts in cats. The average number of platelets per 100× field is multiplied by 20,000.²² Published reference intervals for mean platelet volume (MPV) is reported as 14.2–14.7 fL.^{24,25}

BONE MARROW

Morphology

Marrow samples in the cat are commonly collected from the trochanteric fossa of the femur and the proximal humerus as they are the most accessible sites; however, samples can also be obtained from the iliac crest and sternbra. The morphology of feline hematopoietic cells found in bone marrow aspirates has been reviewed.^{7,9,19,20,21} Morphologic assessment consists of an evaluation of the major hematopoietic cell lines (i.e. erythroid, granulocytic and megakaryocytic) as well as other cells normally found in the marrow (i.e. lymphocytes, plasma cells and macrophages).

Erythroid Series

Stages of nucleated erythroid precursors recognizable in marrow aspirates are rubriblasts, prorubricytes, rubricytes, and metarubricytes. With progressive maturation, the nucleated cells become smaller, undergo nuclear condensation and cytoplasm color changes from deeply basophilic to red-orange. Rubriblasts are present in relatively low numbers. They are a large cell with a high N:C ratio. The nucleus is nearly perfectly round with fine chromatin and one or more visible nucleoli (Fig. 105.13). The cell has moderate amounts of

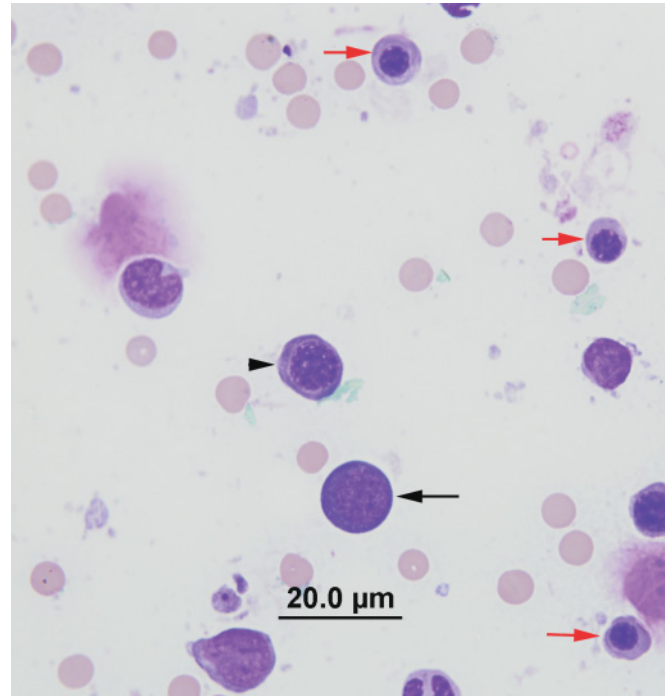


FIGURE 105.13 Feline bone marrow showing developing erythroid cells. A rubriblast (black arrow) with a round nucleus, visible nucleoli and deeply basophilic cytoplasm. A basophilic rubricyte (arrowhead) and three metarubricytes (red arrows). Wright-Giemsa stain.

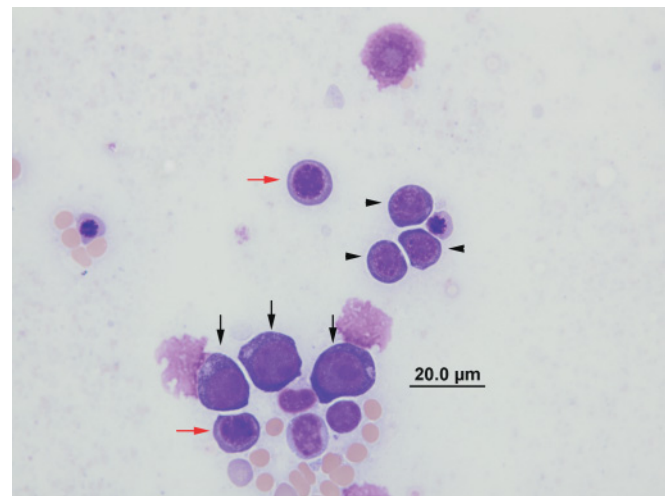


FIGURE 105.14 Feline bone marrow showing early developing erythroid cells. Three rubriblasts (black arrows), three prorubricytes (arrowheads), and two rubricytes (red arrows). Note the chromatin becoming more coarse and condensed as the cell matures. Wright-Giemsa stain.

deeply basophilic cytoplasm. Prorubricytes are slightly smaller, lack visible nucleoli and have somewhat coarser chromatin. The N:C ratio is slightly lower and cytoplasm remains intensely basophilic (Fig. 105.14).

The next stage in maturation is the rubricyte. Rubricytes are decidedly smaller with a lower N:C ratio. The nucleus is smaller and has extremely coarse chromatin; nucleoli are not visible. The cytoplasm undergoes a progressive color change from deeply basophilic to polychromatophilic (blue-orange) to red-orange representing progressive production of hemoglobin. Rubricytes are sometimes separated into three types (basophilic, polychromatophilic, and orthochromic) based predominantly on change in cytoplasm color. Metarubricytes are the smallest of the nucleated

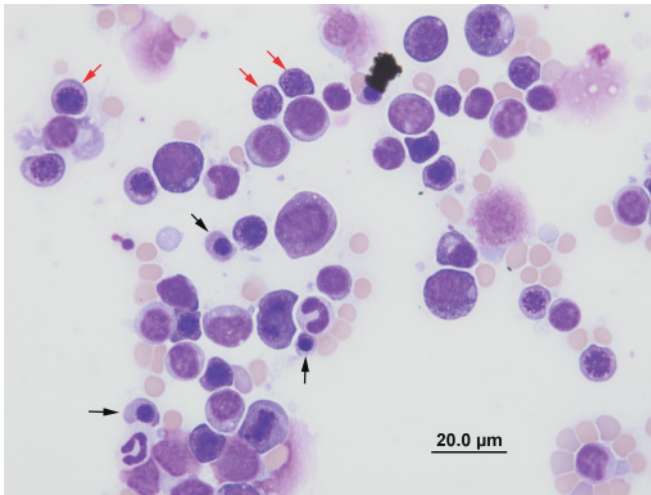


FIGURE 105.15 Feline bone marrow. Developing rubricytes (red arrows) have coarse chromatin. Metarubricytes (black arrows) have condensed pyknotic nuclei. Wright-Giemsa stain.

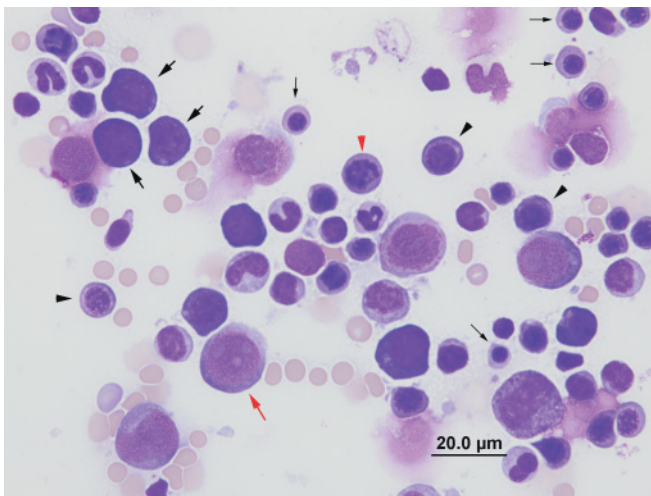


FIGURE 105.16 Feline bone marrow. Developing erythroid cells. Three rubriblasts (large black arrows), rubricytes (black arrowheads), metarubricytes (small black arrows). A few developing myeloid cells—myeloblast (red arrows). A single mitotic figure (red arrowhead). Wright-Giemsa stain.

erythroid precursors and are characterized by a pyknotic nucleus that lacks a discernible chromatin pattern (Figs. 105.15 and 105.16). Progressive maturation results in polychromatophilic erythrocytes, non-nucleated cells similar to those seen in low numbers in the peripheral blood.

Granulocytic Series

The granulocytic series goes through a similar progression of relatively low number of early precursors to more numerous maturing cells. The recognizable stages of granulocytic maturation in the marrow are myeloblasts, promyelocytes (also called “progranulocytes”), myelocytes, metamyelocytes, bands and mature granulocytes.

Myeloblasts are large cells with a high N:C ratio, fine chromatin and one or more visible nucleoli. Compared to rubriblasts, the myeloblast has a more irregularly shaped nucleus and lighter staining cytoplasm. (Fig. 105.16) The next stage in granulocytic development is the promyelocyte stage. Often, these cells are slightly larger than myeloblasts but have a lower N:C ratio due to cytoplasmic enlargement. Nuclear chromatin is somewhat coarser. Nucleoli may or may not be present. The main feature identifying the promyelocytes is the presence of numerous fine, azurophilic, granules termed primary granules in the cytoplasm (Fig. 105.17). These granules help identify the cell as one of granulocytic origin. Myelocytes, smaller than promyelocytes, are characterized by loss of visible primary granules, round to oval nuclei with dense chromatin and no visible nucleoli. Cytoplasm becomes progressively lighter to

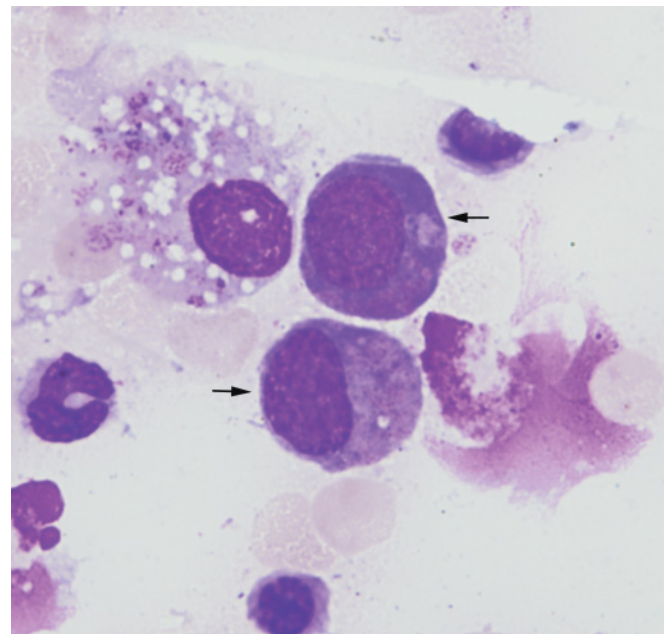


FIGURE 105.17 Feline bone marrow. Two promyelocytes (arrows). Promyelocytes are recognized by the presence of pink-purple primary granules. Wright-Giemsa stain.

clear and secondary granules may be present identifying the cells as eosinophils or basophils (Fig. 105.18). Secondary granules in neutrophilic cells are generally indistinct.

Metamyelocytes are the next stage in maturation and characterized by the beginnings of indentation of the nucleus. The nucleus often has a kidney bean shape. (Fig. 105.19) If the indentation is $>25\%$ of the thickness of the nucleus, then the cell is considered a metamyelocyte, while those with less of an indentation are consid-

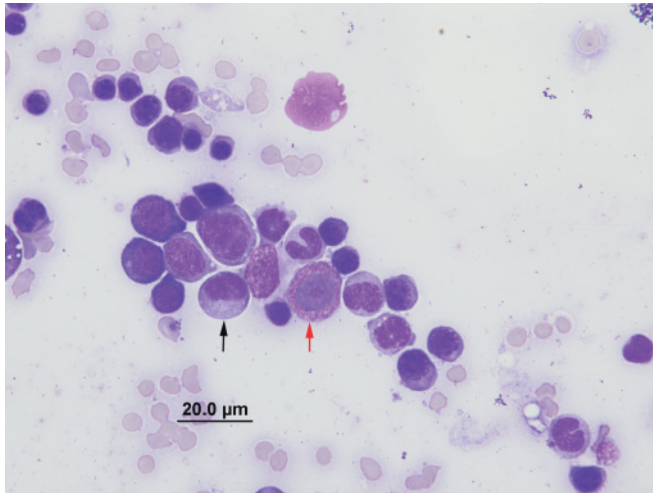


FIGURE 105.18 Feline bone marrow. Developing erythroid and myeloid cells. Neutrophil myelocyte (black arrow) eosinophil myelocyte (red arrow). Wright-Giemsa stain.

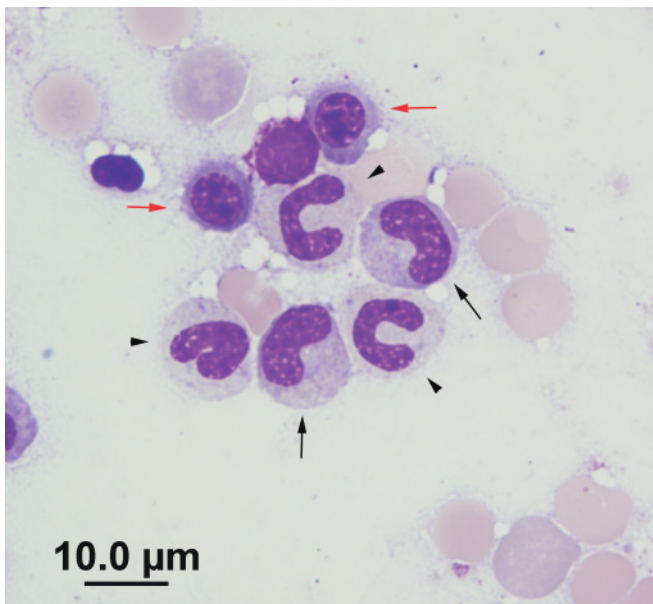


FIGURE 105.19 Feline bone marrow. Developing myeloid cells. Two metamyelocytes (black arrows) and three band neutrophils (black arrowheads). Two rubricytes (red arrows) are also present. Wright-Giemsa stain.

ered myelocytes. Cytoplasm is clear, with the presence of prominent secondary granules in the eosinophilic and basophilic precursors. Progressive indentation of the nucleus gives rise to the “band” cell which has a rod shaped or curved band shaped nucleus with parallel sides and no indentations/constrictions (Fig. 105.19). The final stage is the mature granulocyte characterized by nuclear constrictions giving rise to the classic polylobated appearance. If the nucleus of the cell has any constrictions that are less than half the diameter of the remainder of the nucleus, the cell is characterized as a mature granulocyte. Cytoplasm remains clear in neutrophilic precursors and has well formed granules in the eosinophilic and basophilic cells.

Megakaryocytic Series

Cells of the megakaryocytic series are classified as megakaryoblasts, promegakaryocytes and megakaryocytes. These cells are typically identified on low magnification as most are substantially larger than the other hematopoietic precursors. Megakaryoblasts are present in low numbers. They have a high N:C ratio, single nucleus and deeply basophilic cytoplasm. It is difficult to reliably differentiate them from blasts of other cell lines. Promegakaryocytes are more easily identified. Endomitotic reduplication of the nucleus results in a large cell with 2–4 nuclei (Fig. 105.20). These may appear to be separate or may be seen to be connected by strands of nuclear material. They have more abundant, deeply basophilic cytoplasm than the megakaryoblast. Further maturation with progressive reduplication of the nucleus gives rise to variably sized megakaryocytes that have an enlarging polylobated nuclear mass. As megakaryocytes mature, the cytoplasm becomes more abundant and changes from deeply basophilic to eosinophilic and granular, having an appearance similar to platelets (Figs. 105.21 and 105.22).

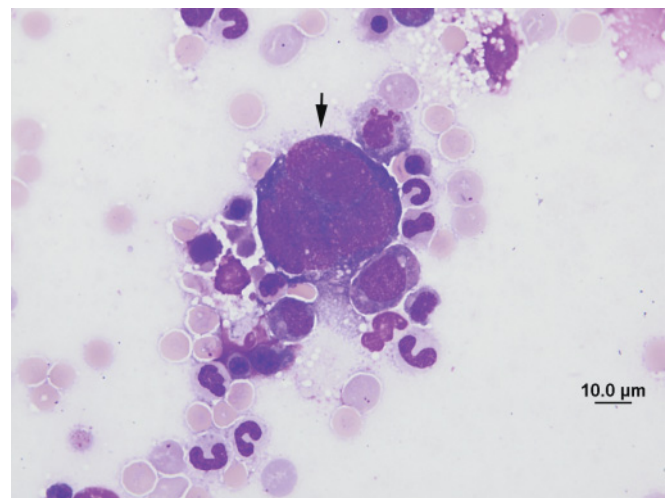


FIGURE 105.20 Feline bone marrow. A promegakaryocyte (black arrow) is recognizable as a large cell with four visible nuclei. Wright-Giemsa stain.

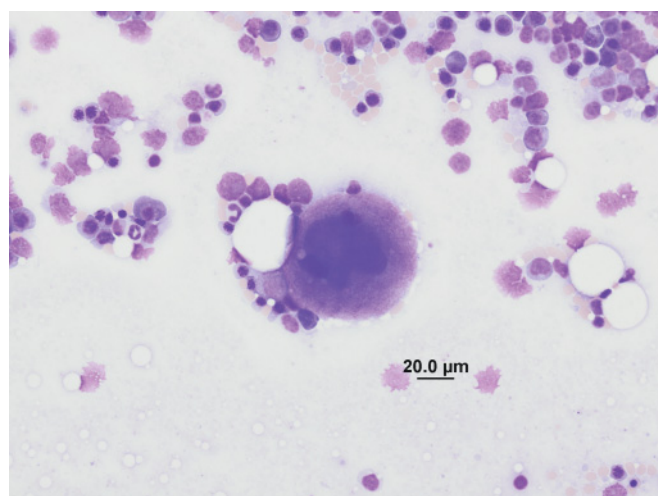


FIGURE 105.21 Feline bone marrow. An early megakaryocyte has a large, convoluted nuclear mass from multiple nuclear reduplications but retains deeply basophilic cytoplasm. Wright-Giemsa stain.

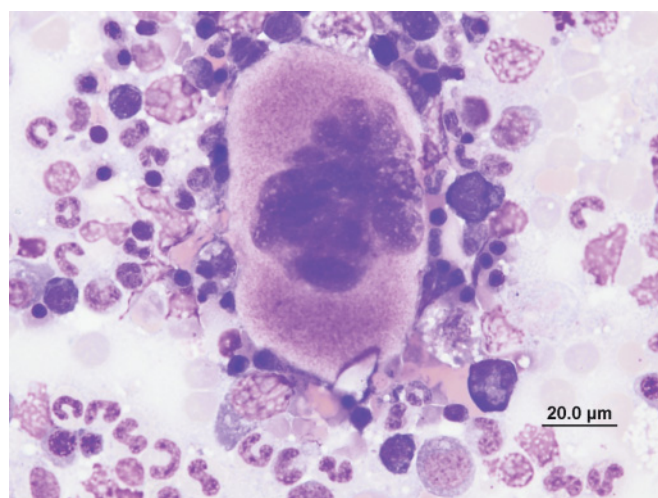


FIGURE 105.22 Feline bone marrow. A mature megakaryocyte is shown with abundant cytoplasm that has pink-purple granules similar to those seen in peripheral blood platelets. Wright-Giemsa stain.

Monocytic and Lymphocytic Cells

Cells of the monocytic series comprise only a small percentage of the cells in normal marrow. Mature monocytes resemble those in peripheral blood. The earlier stage cells, monoblasts and promonocytes, are difficult to clearly separate from granulocytic precursors. Their nuclear shape is more irregular and they lack the primary granules seen in promyelocytes.

Mature lymphocytes may be present in moderate numbers, and low numbers of plasma cells and mature macrophages can be seen admixed amongst the hematopoietic precursors.

TABLE 105.3 Differential Cell Counts from Bone Marrow Samples of Normal Cats

	Gilmore et al. 1964 ^a	Jain 1993 ^b
	Mean (%)	Mean ± SD (%)
Number of cats	15	7
Myeloblasts	1.1	0.08 ± 0.16
Progranulocyte	2.8	1.74 ± 1.04
Myelocyte neutrophil	5.9	4.31 ± 2.49
Myelocyte eosinophil	0.3	0.60 ± 0.42
Metamyelocyte neutrophil	15	10.06 ± 3.20
Metamyelocyte eosinophil	0.2	0.54 ± 0.39
Band neutrophil	14.7	14.4 ± 1.30
Band eosinophil	0.3	0.49 ± 0.40
Neutrophil	14.0	12.86 ± 4.85
Eosinophil	1.3	0.6 ± 0.20
Basophil	NR ^c	0.4
<i>Total myeloid cells</i>	55.6	45.86 ± 3.78
Rubriblast	1.2	0.17
Prorubricyte	2.8	1.0
Rubricyte	16.1	4.02
Polychromatophilic rubricyte	—	17.57
<i>Total erythroid cells</i>	38.4	28.74
Lymphocytes	5.1	16.13
Plasma cells	0.3	0.8
<i>Myeloid:erythroid ratio</i>	1.6:1	1.63:1 ± 0.35:1

^aReference 7.

^bReference 13.

^cNR, not reported.

Quantitative Parameters

The relative proportion of hematopoietic cells found in samples of bone marrow from normal cats is given in Table 105.3. The M:E ratio of feline marrow is generally reported as being between 1.2:1 and 2.0:1.¹³ Bone marrow samples from normal adult cats average less than 10% lymphocytes⁹, but may be as high as 21%.¹³

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Normal Hematology of the Horse and Donkey

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Erythrocytes

- Rouleaux
- Agglutination
- Howell-Jolly Bodies
- Hemoglobin Crystals and Artifacts

Leukocytes

- Neutrophils
- Eosinophils
- Basophils

Lymphocytes

- Monocytes

Platelets

- Breed-Related Differences
- Age-Related Differences
- Gender-Related Differences
- Influences of Training
- Bone Marrow

Acronyms and Abbreviations

CBC, complete blood count; Hct, hematocrit; Hgb, hemoglobin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; M:E ratio, myeloid:erythroid ratio; MPC, mean platelet component; MPV, mean platelet volume; N:L ratio, neutrophil:lymphocyte ratio; nRBC, nucleated red blood cell; PCDW, platelet component distribution width; RBC, red blood cell; RDW, red cell distribution width; WBC, white blood cell.

The common domestic equine species (horses, donkeys, and their hybrid the mule) have various hematological differences as a result of age, breed, sex, reproductive status, emotional status and physical activity. Other differences are minor and observations from ponies and horses are frequently used interchangeably. Complete blood cell count (CBC) reference intervals for horses, neonates and donkeys have been assembled (Tables 106.1 and 106.2); however, it is important to note that these reference intervals have been established for particular methodologies and instruments and as a result they are not applicable to all laboratory methods.

ERYTHROCYTES

The equine red blood cell (RBC) is a biconcave discoid cell that has mild to no central pallor (Fig 106.1A). The cells measure 5–6 μm in diameter and have a lifespan of 140–155 days. Polychromatophilic RBCs are rarely detected in the peripheral blood of horses because equine reticulocytes mature in the bone marrow even when erythropoiesis is increased. Low reticulocyte concentrations have been detected in anemic horses

using an automated hematology analyzer (Advia 120, Bayer Corp., Tarrytown, NY, USA).¹⁰

Rouleaux

Equine RBCs have prominent rouleaux that appear as a stack of coins on a blood film. Rouleaux formation is common in horses because horses have decreased RBC membrane charge (zeta potential) compared to other species.⁵ Increased rouleaux formation can be seen with hyperglobulinemia and hyperfibrinogenemia because these can further mask erythrocyte negative surface charge.⁵ Rouleaux are responsible for the rapid RBC sedimentation rate of equine blood.¹⁹ Thorough mixing of blood specimens is required to prevent erroneous laboratory measurements that result from a rapid sedimentation rate.

Agglutination

Autoagglutination can be seen in some horses without hemolysis as a result of cold antibodies that have maximal activity at 4–20 °C or as a result of unfractionated heparin treatment.^{23,24} Macroscopically, agglutination has a granular appearance and microscopically appears

TABLE 106.1 Reference Intervals for Horses and Donkeys

	Hot-Blooded Breeds ^a	Cold-Blooded Breeds ^b	Miniature Horses ^c	Donkeys ^d	Mixed Breeds ^e
Number of Horses/Donkeys	147	Unknown	47	166	114
Erythrocyte series					
Erythrocytes ($\times 10^6/\mu\text{L}$)	6.8–12.9	5.5–9.5	4.7–9.4	4.7–9.0	6.0–11.3
Hemoglobin (g/dL)	11.0–19.0	8.0–14.0	8.9–16.3	9.5–16.5	10.6–18.9
Hct (%)	32–53	24–44	24–44	28–47	34–49
MCV (fL)	37–59	—	37–58	46–67	38–49
MCH (pg)	12–20	—	13–23	16–23	15–19
MCHC (g/dL)	31–39	—	35–40	32–36	37–40
RBC diameter (μm)	5–6	—	—	—	—
RDW (%)	24–27	—	—	—	17–21
Leukocyte series					
Total leukocytes ($/\mu\text{L}$)	5400–14,300	6000–12,000	5010–14,970	5400–15,500	5290–13,750
Neutrophil (band)	0–1000	—	—	0–100	—
Neutrophil (segmented)	2260–8580	—	—	2200–10,100	2700–9650
Lymphocytes	1500–7700	—	—	1100–7400	1100–5690
Monocytes	0–1000	—	—	70–1200	130–590
Eosinophils	0–1000	—	—	0–1719	60–580
Basophils	0–290	—	—	0–190	10–160
Percentage distribution					
Neutrophil (band) (%)	0–8	0–2	0–1.6	0–0.7	—
Neutrophil (segmented) (%)	22–72	35–75	21–53	23–69	—
Lymphocytes (%)	17–68	15–50	41–77	19–67	—
Monocytes (%)	0–14	2–10	0–2	0–11	—
Eosinophils (%)	0–10	2–12	0–7	0–14	—
Basophils (%)	0–4	0–3	0–2	0–1.4	—
Other data					
Plasma proteins (g/dL)	5.8–8.7	—	—	6.0–8.4	—
Fibrinogen (g/dL)	0.1–0.4	—	—	0.1–0.5	—
Platelet ($\times 10^3$)	100–350	—	—	160–584	46–194
CHCM ^f (g/dL)	—	—	—	—	37–40
CH ^f (pg)	—	—	—	—	15–19
HDW ^f (%)	—	—	—	—	2–3
Reticulocytes ($\times 10^3$)	—	—	—	—	0.5–85.1
CHCM ^r (fL)	—	—	—	—	27–39
CH ^r (pg)	—	—	—	—	14–23
MCV ^r (fL)	—	—	—	—	37–73
MPV (fL)	—	—	—	—	5.6–8.3

^{a,b}Adapted from Jain NC. Schalm's Veterinary Hematology. 4th ed. Philadelphia: Lea & Febiger, 1986. These reference intervals were determined by unknown methods.

^cAdapted from Harvey RB, Hambright MB, Rowe LD. Clinical biochemical and hematologic values of the American Miniature Horse: reference values. Am J Vet Res 1984;45:987–990. These reference intervals were determined on a Model ZBI and Coulter hemoglobinometer (Coulter Electronics Inc, Hialeah, FL).

^dAdapted from Zinkl JG, Mae D, Guzman Merida P, et al. Reference ranges and the influence of age and sex on hematologic and serum biochemical values in donkeys (*Equus asinus*). Am J Vet Res 1990;51:408–413. These reference intervals were determined by unknown methods.

^eAdapted from Giordano A, Rossi G, Pieralisi C, et al. Evaluation of equine hemograms using the ADVIA 120 as compared with an impedance counter and manual differential count. Vet Clin Pathol 2008;37:21–30. Reference intervals were determined from an ADVIA 120 (Bayer Corporation, Tarrytown, NY) and represent those between 2.5 and 97.5 percentile values.

^fCH, red cell hemoglobin content; CHCM, red cell hemoglobin concentration mean; CHCM^r, reticulocyte hemoglobin concentration mean; CH^r, reticulocyte hemoglobin content; HDW, hemoglobin concentration distribution width; MCV^r, mean reticulocyte volume.

as grape-like clusters of RBCs. Rouleaux and agglutinated erythrocytes can be difficult to differentiate in a blood film. Agglutination can be differentiated from rouleaux by performing a saline dilution test. Typically a 1:2 dilution will disperse rouleaux but not the autoagglutinated erythrocytes. Infrequently, a higher dilution (up to 1:10) may be needed to disperse rouleaux. Agglutination can cause erroneous MCV values and erythrocyte concentrations determined by impedance because the aggregates may interfere with the electronic or optical evaluation of the erythrocytes. Pre-treating

cell suspensions from agglutinated heparin treated horses with trypsin can reverse the agglutination and improve the accuracy of cell counts and indices.²⁴

Howell-Jolly Bodies

Howell-Jolly bodies are nuclear remnants that occasionally can be seen in equine peripheral blood films in health (Fig. 106.1A). They are small, round, purple inclusions found in discocytes. Increased numbers of Howell-Jolly bodies can be seen with increased eryth-

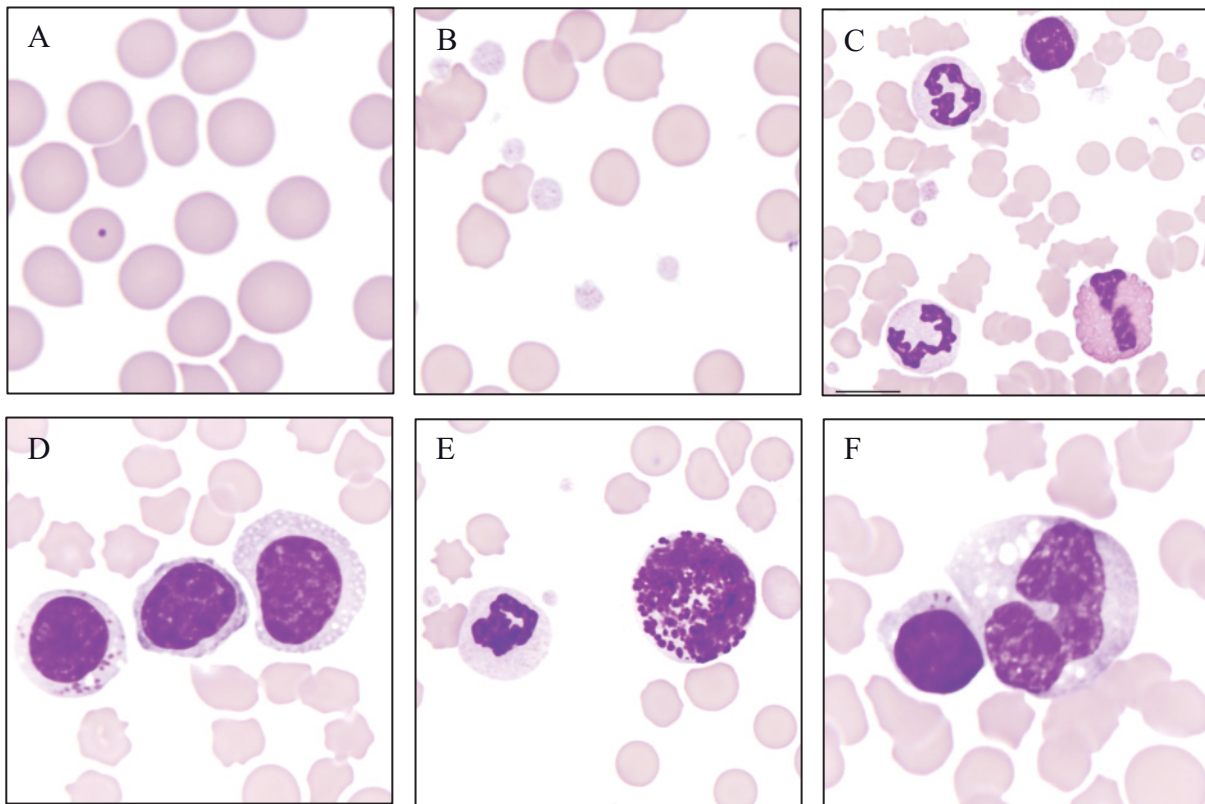


FIGURE 106.1 (A–F) Peripheral blood film from a horse. Modified Wright’s stain: (A) Discocytes that lack a central pallor and an erythrocyte that contains a Howell-Jolly body. (B) Six light-staining platelets. (C) Two segmented neutrophils, one small lymphocyte, one eosinophil, erythrocytes and platelets. (D) Three lymphocytes and erythrocytes; small lymphocyte that contains cytoplasmic granules (left), small lymphocyte with scant cytoplasm (center), small lymphocyte with slightly more cytoplasm (right). (E) Segmented neutrophil, basophil, erythrocytes and platelets. (F) Small lymphocyte (with azurophilic granules), monocyte and erythrocytes.

ropoiesis and with decreased or compromised splenic function.

Hemoglobin Crystals and Artifacts

Hemoglobin crystals are rarely seen in peripheral blood films in health and their significance has not been determined to date. Refractile artifacts can be seen and develop as a result of the drying or staining process.

LEUKOCYTES

Automated determination of differential leukocyte counts has been attempted on several hematology instruments using species-specific programs, but to date no methods have been found to be completely reliable.^{2,13,26,29} Therefore, it is still recommended that leukocyte differential cell counts be verified by examination of a peripheral blood film.

Neutrophils

The nuclei of equine neutrophils are lobulated with scalloped to jagged nuclear membranes and have heavily plaqued chromatin (Fig 106.1C). Nuclear filaments that separate lobes are rarely seen.¹⁹ Barr bodies

(sex chromatin lobe/drumstick) can be recognized in females and resemble a small purple body attached to the nucleus by a thin chromatin strand. The neutrophil cytoplasm is colorless with granules that may or may not stain depending on the type of stain and stain conditions used. Equine band neutrophils are less frequently seen because horses do not exhibit marked left shifts in inflammatory states compared to other species (i.e. dog and cat). Because of this, hyperfibrinogenemia is frequently used as an additional indicator of inflammation.

Hypersegmented neutrophils are rarely seen in health and historically are recognized by having five or more lobes separated by filaments. Prolonged storage of blood may lead to the artifactual development of hypersegmented leukocytes. Idiopathic hypersegmentation has been found in Quarter Horses that lacked evidence of clinical disease.²⁷

Hyposegmented neutrophils have been reported in apparently healthy Arabian horses that were diagnosed with Pelger-Huët anomaly.¹⁴

Eosinophils

Eosinophils contain large, reddish-orange, specific granules that tightly pack the cytoplasm, often obscuring the nuclei and giving a raspberry-like appearance

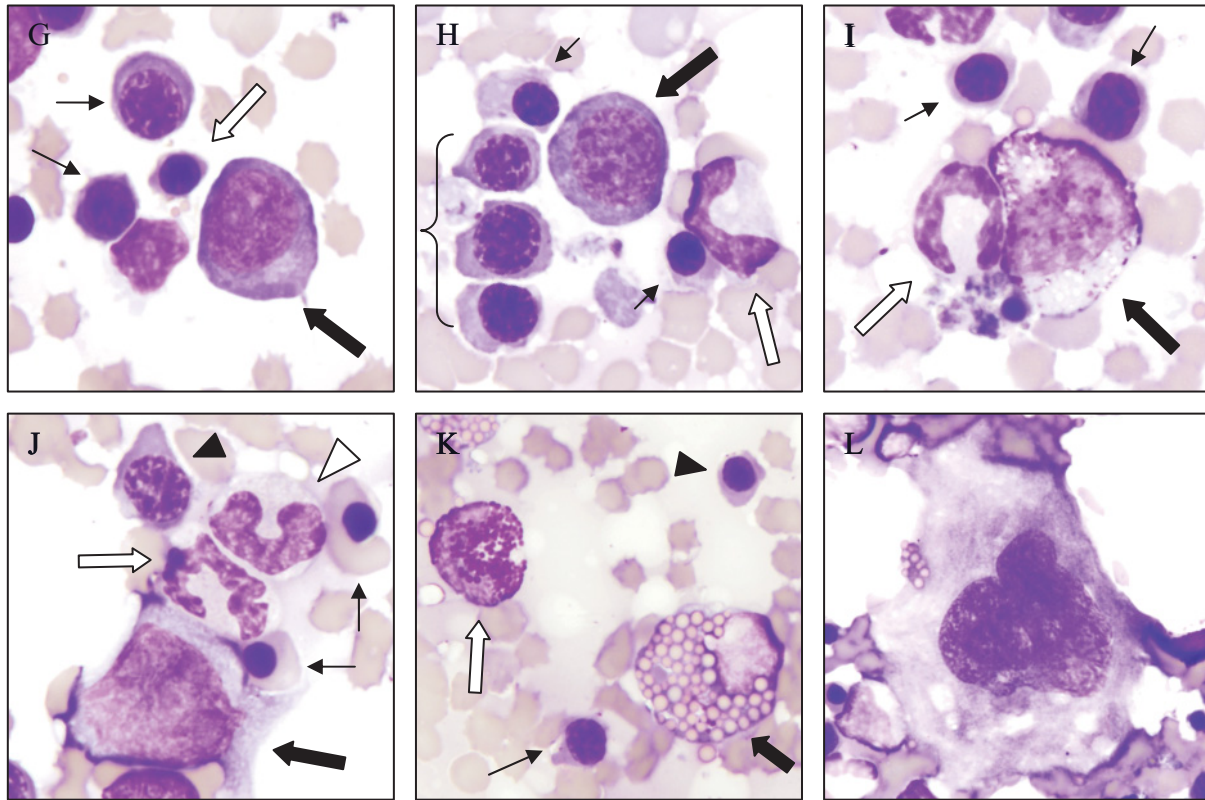


FIGURE 106.1 *continued* (G–L) Bone marrow preparation from a horse. Modified Wright's stain: (G) Rubriblast (dark thick arrow), two rubricytes (thin black arrows) and one metarubricyte (thick white arrow). (H) Prorubricyte (dark thick arrow), three rubricytes (bracket), two metarubricytes (small thin black arrows), one band, (thick white arrow). (I) Progranulocyte (thick black arrow), segmented neutrophil (thick white arrow), two rubricytes (thin black arrows). (J) Myeloblast (thick black arrow), band (white arrow head), segmented neutrophil (white thick arrow), rubricyte (black arrow head) and two metarubricytes (thin black arrows). (K) Eosinophil progranulocyte (thick black arrow), basophil metamyelocyte (white thick arrow), rubricyte (thin black arrow), metarubricyte (arrowhead). (L) Megakaryocyte.

(Fig. 106.1C). Their cytoplasm is pale blue. The lobulated nucleus seldom shows fine filamentation. Degranulated eosinophils are vacuolated and are rarely seen in health.

Basophils

Equine basophils contain few to many purple granules that vary in size and shape (Fig 106.1E).¹⁹ When granules are abundant, they mask the nucleus. The nuclei of basophils are less segmented compared to the nuclei of mature neutrophils.¹⁹ Cytoplasm varies from clear to light blue.

Lymphocytes

Lymphocytes are made up of 38–66% T cells, 17–38% B cells with the remainder being null cells.^{22,33} Lymphocytes are smaller than granulocytes with a dark-staining nuclei, coarse chromatin patterns and scant amounts of blue cytoplasm (Fig. 106.1D). On Wright-stained blood films, about 5% of the lymphocytes contain small, magenta, irregularly-shaped, cytoplasmic granules (Fig. 106.1D). Occasionally, larger lymphocytes are present that have smooth chromatin patterns and large

amounts of pale blue cytoplasm.¹⁹ Reactive lymphocytes (immunocytes) are rarely seen in health. They are slightly larger than small lymphocytes, have scalloped nuclear margins, moderately aggregated chromatin, occasionally have discernible nucleolar rings, scant to moderate amounts of intensely basophilic cytoplasm, and occasionally have a pale-staining Golgi zone.

Monocytes

The nucleus of the equine monocyte is large, broad, variable in shape (i.e. oval, bilobed, horseshoe-shaped, trilobed or irregular) with lacy chromatin (Fig. 106.1F). Cytoplasm is gray-blue, has small azurophilic granules and may have a few clear vacuoles. Prolonged storage of blood has resulted in an artifactual increase in the proportion of mononuclear cells detected by the Advia 120 hematology analyzer (Bayer Corp., Tarrytown, NY, USA).⁹

PLATELETS

Equine platelet concentrations are some of the lowest reported for mammals (Table 106.1).⁴ Reported reference intervals for different horse breeds have been

summarized elsewhere.¹⁹ Finding 6–10 platelets/oil-immersion field (100× objective) in a peripheral blood film indicates an adequate platelet concentration. Equine mean platelet volume (MPV) (4.3–5.6 fL) and mean platelet mass ($0.47\text{--}0.96 \times 10^6/\text{fL}$) have been determined.⁴ Additionally, mean platelet component (MPC) concentration ($26.2 \pm 3.5 \text{ g/dL}$) and platelet component distribution width (PCDW) ($7.9 \pm 1.2 \text{ g/dL}$) have been summarized.³² MPC was found to decrease with disease processes that caused activation of platelets. Equine platelets stain very lightly with Wright-Giemsa stain and sometimes can be difficult to discern on blood films. However, platelets stain well with Diff-Quick® stain. Platelets are round, oval, or elongate, measure 2.5–3.5 μm in diameter, and have light blue cytoplasm that contains fine azurophilic granules (Fig. 106.1B). Pseudopodia may be noted with activated platelets. Giant platelets (greater than the diameter of an RBC) are associated with increased thrombopoiesis. Platelet clumping indicates platelet activation and aggregation during blood collection, and can lead to erroneously low platelet concentrations. Prolonged sample storage can result in pseudothrombocytosis due to misclassification of ghost RBCs as platelets with the Advia 120 (Bayer Corp., Tarrytown, NY, USA).⁹ EDTA-dependent pseudothrombocytopenia has been reported in a Thoroughbred gelding.¹⁸

BREED-RELATED DIFFERENCES

Horses of Arabian ancestry (light-horse breeds) are called “hot-blooded” and include Arabian and Thoroughbred breeds. “Cold-blooded” horses are the draft horse and pony. Most horse breeds fall into the “warm-blood” category which are crosses between hot-bloods and cold-bloods (Standardbreds and Quarter Horses). Hot-blooded horses have been found to have higher RBC counts, hemoglobin (Hgb) concentration, hematocrit (Hct), and blood volume compared to cold-blooded horses.¹⁹ Hcts as low as 24% have been found in healthy draft horses and pony breeds. Thoroughbreds have a smaller RBC mean corpuscular volume (MCV) than draft horses.¹⁹ Specific breeds have minor differences in Hgb, mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC); however, these differences are insignificant.¹⁹ American miniature horses have lower Hct, Hgb, and RBC counts, but higher MCV, MCH and MCHC.¹⁷ Reference intervals for different breeds of donkeys have been established and compared.^{7,11,12,25,35} Donkeys have similar RBC counts, Hgb and Hct compared to ponies; however, donkeys were found to have much higher MCV.²⁰

Minor differences have been found among various breeds in relation to WBC count, with hot-blooded horses having slightly higher WBC counts compared to cold-blooded horses.¹⁹ Thoroughbreds and Arabians have a mean neutrophil:lymphocyte (N:L) ratio of 1.0 whereas cold-blooded horses have a mean N:L ratio of 1.7.¹⁹ Miniature horses have a N:L ratio of 0.67.¹⁷

AGE-RELATED CHANGES

Red blood cell parameters (i.e. Hct, RBC count and Hgb) are increased at birth, decline sharply within 12–24 hours, then decline gradually over the subsequent 2 weeks where they remain in the lower portion of the normal adult reference interval during the first year of life (Table 106.2).^{15,19} The initial hematologic changes in foals at birth are thought to be due to the transfusion of placental blood to the foal. The sharp decline of RBC values in the first 12–24 hours of life have been attributed to catecholamine secretion and adjustment of fluid balances as a result of the osmotic effect of absorption of colostral immunoglobulins. Further decline in these values has been attributed to decreased RBC survival time, decreased iron delivery to bone marrow, decreased stimulus for erythropoietin production as a result of higher Hgb saturation, increased blood oxygen content, and enhanced delivery of oxygen to the tissues due to lower 2,3-diphosphoglycerate concentrations.¹⁵ Normal adult hematologic values are attained at 1–2 years of age. A similar trend of declining erythroid parameters during the first year has also been noted in donkeys.⁶

Mean cell volume values are high at birth and then decrease, reaching their lowest values at 3–5 months of age (see Table 106.2).^{15,19} They do not increase to adult values until approximately 1 year of age.¹⁵ Microcytosis has been attributed to a decrease in serum iron as a result of increased growth demand.¹⁶ These RBCs may be too small to be recognized as erythrocytes by some impedance counters, hence generating false MCV, RBC, and Hct values. Mild anisocytosis is a typical finding in young foals.¹⁵ The MCHC remains constant after birth and is similar to adult values.¹⁵ Donkey foals between 6 and 12 months of age have higher MCV (44–52 fL) values compared to horse foals.²⁰

Neutrophil numbers are low in the fetus (<1,500/μL, before 300 days of gestation), increase after birth in response to cortisol (8,000/μL), and then decrease to mean adult values (4,000/μL) at about 4–6 months of age.^{1,15,19} Band neutrophils do not exceed 150/μL in healthy foals.¹⁵ Foals born at term have higher neutrophil concentrations than foals born prematurely. In donkeys, neutrophil numbers decreased from birth to 10 months of age and then increased to adult reference intervals by 18 months of age.⁶ Lymphocyte numbers in foals are low at birth (average 1,400/μL), increase to 5,000/μL at 3 months of age, and reach adult values at 1 year of age.^{15,19} Consequently, N:L ratios have been found to decrease from 2.8 at birth to 1.0 at 1 year of age; a similar trend is seen in donkeys.^{6,19} Lymphocyte numbers further decline during adulthood while neutrophil numbers remain the same, resulting in a higher N:L ratio in aged horses compared to foals.¹⁹ Eosinophils are not routinely detected in the fetus and foals at birth, and reach a mean of 400/μL by 4 months of age.¹⁵ In donkeys, eosinophil numbers were found to increase at a relatively constant rate up to 2 years of age.⁶ Monocyte numbers remain constant and do not change with age.¹⁵

TABLE 106.2 Reference Intervals for Thoroughbred and Quarter Horse Foals of Both Sexes (mean \pm 1 S.D.)^a

	1st Day	2–7 Days (Average 5)	8–14 Days (Average 9)	21–30 Days (Average 28)	1–3 months (Average 51 days)
Number of foals	34	16	15	8	14
RBCs ($\times 10^6/\mu\text{L}$)	10.5 \pm 1.4	9.5 \pm 0.8	9.0 \pm 0.8	11.2 \pm 1.3	11.9 \pm 1.3
Hgb (g/dL)	14.2 \pm 1.3	12.7 \pm 0.9	11.8 \pm 1.2	13.1 \pm 1.1	13.4 \pm 1.6
PCV (%)	42 \pm 3.6	37 \pm 2.8	35 \pm 3.7	38 \pm 3.3	38 \pm 4.1
MCV (fL)	40 \pm 4	39 \pm 3	39 \pm 2	34 \pm 2.4	32 \pm 2
MCH (pg)	14 \pm 1	13 \pm 1	13 \pm 1	12 \pm 1	11 \pm 1
MCHC (%)	34 \pm 2	34 \pm 1	34 \pm 1	35 \pm 1	35 \pm 1
Plasma proteins (g/dL)	6.2 \pm 0.9(32)	6.4 \pm 0.5	6.1 \pm 0.6	6.2 \pm 0.4	6.4 \pm 0.4
Fibrinogen (mg/dL)	270 \pm 60(15)	330 \pm 130(6)	300 \pm 50(9)	400 \pm 50(5)	460 \pm 70(10)
Total leukocytes ($/\mu\text{L}$)	9602 \pm 3372	9300 \pm 2346	9483 \pm 2196	9688 \pm 1940	10,893 \pm 2977
Neutrophils (band)	138 \pm 198	29 \pm 37	48 \pm 125	19 \pm 33	10 \pm 28
Neutrophils (segmented)	6824 \pm 2757	6448 \pm 2128	6338 \pm 1849	5501 \pm 1346	5315 \pm 2437
Lymphocytes	2192 \pm 891	2420 \pm 739	2633 \pm 933	3823 \pm 863	5086 \pm 1419
Monocytes	414 \pm 373	308 \pm 172	302 \pm 124	266 \pm 192	348 \pm 175
Eosinophils	0	30 \pm 34	21 \pm 38	48 \pm 53	115 \pm 88
Basophils	14 \pm 78	41 \pm 44	29 \pm 50	11 \pm 29	12 \pm 26
Leukocytes (%)					
Neutrophils (band)	1.5 \pm 1.8	0.3 \pm 0.4	0.5 \pm 1.1	0.2 \pm 0.3	0.1 \pm 0.3
Neutrophils (segmented)	68.9 \pm 10.7	68.2 \pm 9.4	66.2 \pm 9.0	56.8 \pm 7.4	46.9 \pm 12.1
Lymphocytes	25.1 \pm 10.3	27.0 \pm 9.8	28.5 \pm 9.4	39.6 \pm 6.5	48.5 \pm 11.5
Monocytes	3.9 \pm 2.9	3.4 \pm 1.9	3.3 \pm 1.5	2.6 \pm 2.0	3.3 \pm 1.8
Eosinophils	0	0.3 \pm 0.4	0.2 \pm 0.4	0.4 \pm 0.5	1.0 \pm 0.8
Basophils	0.02 \pm 0.08	0.4 \pm 0.4	0.3 \pm 0.5	0.1 \pm 0.3	0.1 \pm 0.3
Neutrophil:lymphocyte ratio	2.8:1	2.5:1	2.3:1	1.4:1	1.1:1

^aAdapted from Jain NC. Schalm's Veterinary Hematology. 4th ed. Philadelphia: Lea & Febiger, 1986. Numbers in parentheses indicate number of foals when less than total for series. Methods used for determination of these reference intervals are unknown.

Platelet numbers in foals appear to be constant over the first year of life and do not appear to be influenced by age.¹⁵ Equine MPV (range 4.8–6.1 fL) and mean platelet mass (range 0.62–1.29 $\times 10^3/\mu\text{L}$) have been determined in foals at 24–48 hours of age.⁴ Additionally, MPC concentration (28.1 \pm 1.7 g/dL) and PCDW (6.6 \pm 0.8 g/dL) have been determined for foals less than 21 days of age.³² MPCs were found to be higher in neonates compared with adult horses.³² In donkeys, platelet concentration increases in the first month of life and then remains within adult reference intervals.⁶

Total plasma protein concentration increases from 5 to 6 g/dL after birth due to immunoglobulins absorbed from colostrum.¹⁵ Fibrinogen concentration is low at birth (about 200 mg/dL) and increases to 500 mg/dL by 3 months of age.¹⁵

Hematologic values have been evaluated in equine fetuses (202 and 238 days of gestation) and consist of lower RBC count, Hgb, Hct, plasma protein, fibrinogen and white blood cell (WBC) count; higher MCV, MCH, increased numbers of nucleated RBC, Heinz bodies, and Howell-Jolly bodies, marked polychromasia; mean N:L ratio of 0.2; and no difference in platelet concentration and MCHC when compared with previously published reference intervals for foals.¹

Hematologic data were compared among young horses (<5 years of age) and geriatric horses (>20 years

of age).²⁸ Geriatric horses were found to have consistently larger RBCs; however, the MCV still remained within the reference interval for the normal horse population. Hct and Hgb did not differ significantly. When comparing donkeys of various age groups (<1 year of age to >6 years of age); RBC, lymphocyte, platelet, and fibrinogen concentrations were lower in older donkeys, while eosinophil concentrations, MCV values, MCH values, and plasma protein concentrations were greater in older donkeys.³⁵

GENDER-RELATED DIFFERENCES

Minor differences between adult females and adult males have been reported. Males had slightly higher RBC counts, Hgb and Hct, while females had higher MCH, MCHC, and WBC values.¹⁹

Mares in late pregnancy and during the first few weeks postpartum have lower Hct, RBC count, and Hgb, which has been attributed to a hemodilution effect resulting from increased plasma volume. Lactating mares also tend to have lower mean values for Hct, RBC count, and Hgb.¹⁹ Female donkeys were found to have higher MCH, and leukocyte and neutrophil counts compared to males in one study;³⁵ while others found no differences between genders.^{11,12}

TABLE 106.3 Cellular Composition of the Bone Marrow of Horses

Cell Type	Archer (1954) (12 horses)		Calhoun (1954) (7 horses)		Tschudi et al. (1975) (15 horses)		Franken et al. (1982a) (24 horses)	
	Range (%)	Mean (%)	Range (%)	Mean (%)	Range (%)	Mean (%)	Range (%)	Mean (%)
Myeloblast	0.7	0.2	—	—	0.3–2.0	1.2	0–5.0	1.0
Promyelocyte	0.1–1.0	0.5	0.0–5.0	1.8	0.0–3.0	1.3	0.5–3.5	1.7
Myelocytes								
Neutrophilic	11.1–28.6	17.9	26.2–56.0	38.1	1.0–5.0	3.3	1.0–7.5	3.2
Eosinophilic	0.7–2.4	1.4	0.4–3.6	2.3	0.0–0.3	0.1	—	—
Metamyelocytes								
Neutrophilic	17.9–29.4	21.8	—	—	5.0–11.0	7.91	1.5–15.0	5.6
Eosinophilic	1.1–7.0	3.5	—	—	0.0–0.3	0.1	—	—
Basophilic	0.1–5.4	1.0	—	—	0.0–0.3	0.1	—	—
Band neutrophils	—	—	—	—	—	—	6.0–26.5	15.7
Mature granulocytes								
Neutrophils	7.8–25.3	14.7	1.8–20.2	13.3	11.0–30.0	20.4	3.0–16.5	8.4
Eosinophils	0.5–6.0	2.6	0.2–1.2	0.6	0.0–0.6	0.2	0.0–5.0	1.8
Basophils	0.0–0.3	0.1	0.0–1.0	0.6	0.0–0.5	0.1	0.0–1.0	0.3
<i>Total myeloid series</i>		63.7		56.7		34.5		37.9
Rubriblast	0.0–0.9	0.3	0.4–3.4	1.6	0.6–4.0	2.2	0.0–2.0	0.7
Prorubricyte	0.2–4.4	1.9	—	—	2.0–9.0	5.8	1.0–9.5	3.6
Rubricyte	2.2–13.0	6.3	8.0–32.0	20.9	10.0–23.0	16.2	14.5–44.0	28.2
Metarubricyte	4.2–39.2	17.6	5.0–24.2	13.7	25.0–45.0	34.9	14.0–36.0	23.2
<i>Total erythroid series</i>		26.2		34.7		60.5		55.9
Monocyte	—	—	1.2–4.8	2.5	0.0–2.0	0.8	0.0–1.0	0.2
Lymphocyte	2.5–20.9	9.7	2.0–5.6	3.9	1.0–6.0	3.8	1.5–8.5	3.8
Plasma cell	0.0–1.7	0.5	0.0–0.8	0.6	0.0–2.0	0.7	0.0–2.0	0.6
Megakaryocyte	—	—	—	—	—	—	0.0–1.0	0.3
Mitotic figures	—	—	—	—	0.4–2.0	1.2	0.0–3.5	0.8
<i>Myeloid:erythroid ratio</i>	1.1–10.2	2.4	0.9–3.8	1.6	0.3–0.9	0.6	0.5–0.9	0.7

Adapted from Jain NC. Schalm's Veterinary Hematology. 4th ed. Philadelphia: Lea & Febiger, 1986.

INFLUENCES OF TRAINING

Assessment of training-related issues is difficult as they are influenced by the breed of horse, demeanor of the horse, age of horse, amount of training the horse has received, when the sample was taken in relation to exercise, intensity of exercise, and type of event in which the animal is engaged.^{3,30,31} In spite of these variables, intense exercise is associated with transient RBC changes including increased Hgb (up to 23 g/dL), Hct (up to 65%) and RBC count.³ These erythrocyte changes are attributed to epinephrine release, an increase in sympathetic tone, splenic contraction and fluid shifts out of the vascular compartment.^{3,30} The degree of change and duration of elevations depend on the degree of training and intensity of exertion (values return to normal within 30 minutes after light exercise and up to 3 hours after hard exertion).³ The effect of training on resting hematologic values has been variable and reports of no changes to decreases in erythrocyte parameters have been reported.^{8,31} A relationship between hemograms, racing performance and fitness level has not been found.^{30,31} Exertion-induced leukogram changes have been variable, and a leukocytosis charac-

terized by a neutrophilia and lymphocytosis, or leukocytosis due to neutrophilia and lymphopenia have been reported and appear to depend on the type and intensity of exercise.^{3,8} These leukogram changes have been attributed to epinephrine and cortisol release (also see Chapter 47).³ Regular training has not produced any differences in baseline total leukocyte concentration or baseline differential leukocyte concentrations.^{3,31}

BONE MARROW

Persistent anemia, leukopenia, thrombocytopenia, or hematopoietic neoplasms are the most frequent indications for examining bone marrow in the horse. Bone marrow can be collected from the sternum (preferred site), ilium and ribs in both adult and young horses as well as from the tuber coxae in young horses. Care must be taken when collecting samples from the sternum and ribs in order to avoid inadvertent penetration of the thoracic cavity. Detailed descriptions on collection techniques have been described (see Chapter 132).²¹ Descriptions of the cellular composition of horse bone marrow have been published (Table 106.3;

Fig 106.1G–L).^{21,34} Cellularity of horse bone marrow can be routinely assessed on bone marrow aspirates or bone marrow core biopsy specimens. Normocellular particles in the adult horse are typically composed of 50% fat and 50% cells but can vary based on the age of the horse.²¹ Young horses can have up to 70% hematopoietic cells to compensate for normal cell turnover and in response to growth. Aged horses can have up to 70% fat. Thorough examination of the bone marrow involves evaluation of the megakaryocytes, including assessment of megakaryocyte numbers and maturity. Typically, one to several megakaryocytes are seen per 10× objective field of view in health; greater than 10–20 per 10× objective field of view are considered increased, and only finding a few after examination of multiple particles suggests decreased numbers.²¹ Maturation of the erythroid series should be orderly, with approximately 5% of the erythroid series consisting of rubriblasts and prorubricytes and 95% of the series consisting of rubricytes and metarubricytes.²¹ Maturation of the granulocytic series should be orderly, with approximately 85% of the granulocytes being metamyelocytes, bands or segmented cells and 15% being myeloblasts and progranulocytes.²¹ The M:E ratio varies from 0.5 to 2.4. This large range limits the use of M:E as an indicator of accelerated erythropoiesis. Other cells present in health are small lymphocytes (2–9%), plasma cells (<2%), monocytes/macrophages (<1%), mitotic figures (<4%) and rarely osteoclasts or osteoblasts.²¹ Stainable bone marrow iron should easily be found in health and the amount increases with age in horses.

Evaluating accelerated erythropoiesis in horses can be difficult to assess because polychromatophils are rarely present in peripheral blood films and reticulocytes are not detected by most hematology analyzers. Finding more than 5% reticulocytes in the bone marrow (demonstrated by methylene blue staining) supports a regenerative response. In intense regenerative responses, bone marrow reticulocyte percentages can go as high as 60%. Caution should be used because blood-contaminated bone marrow will have a falsely low marrow reticulocyte count.

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Normal Hematology of Cattle

DARREN WOOD and GERARDO F. QUIROZ-ROCHA

Erythrocytes

- Erythrocyte Morphology
- Erythrocyte Membrane
- Hemoglobin
- Erythrocyte Metabolism
- Erythrocyte Development

Leukocytes

- Neutrophils

Eosinophils

- Basophils

- Lymphocytes

- Monocytes

Platelets

- General Physiologic and Environmental Influences

- Bone Marrow

- Plasma Proteins

Acronyms and Abbreviations

BLV, bovine leukemia virus; CBC, complete blood count; 2,3 DPG, 2,3 diphosphoglycerate; GPX, glutathione peroxidase; HgbA, adult hemoglobin; HgbE, embryonal hemoglobin; HgbF, fetal hemoglobin; Hgb, hemoglobin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MetHgb, methemoglobin; M:E ratio, myeloid to erythroid ratio; OxHgb, oxyhemoglobin; PC, phosphatidylcholine; RBC, red blood cell; RDW, red cell distribution width; MPV, mean platelet volume; SM, sphingomyelin; WBC, white blood cell.

ERYTHROCYTES

Erythrocyte Morphology

The mature red blood cell (RBC) of the adult bovine is biconcave in shape, has a width of 5–6 μm , has minimal central pallor and a relatively long lifespan of approximately 130 days.³⁷ Anisocytosis is mild to moderate in ruminants. Red blood cell shape is relatively uniform, but poikilocytosis is not unusual in blood smears from apparently healthy calves. This may reflect a unique hemoglobin molecule, its association with the RBC membrane, or inadequate iron stores.³³ Rouleaux formation of bovine RBCs is unusual unless inflammatory proteins are increased.

Polychromatophils are generally absent from the blood of normal adult cattle. However, when severe anemia occurs, reticulocytosis appears in peripheral blood in association with macrocytosis, polychromasia, and basophilic stippling of RBCs, indicating accelerated erythropoiesis. Basophilic stippling is not uncommon during accelerated erythropoiesis in ruminants and should be interpreted as evidence of a regenerative response.

Erythrocyte Membrane

The bovine RBC membrane (and that of other ruminants) is unique with respect to phospholipid composition in the external hemilayer. Instead of a predominance of phosphatidylcholine (PC), sphingomyelin (SM) is present in higher concentration.¹⁰ This is thought to be due the presence of anti-PC antibodies, formed due to evolutionary pressure of coexisting rumenal ciliates whose membranes contain a similar analogue.¹³ This variation may have an impact on the relatively low occurrence of rouleaux formation in this species.³⁵

Hemoglobin

Ruminant hemoglobins are of particular interest because of the large amount of polymorphism that occurs between species, breeds, and even within the individual as it develops from embryo to adult.²¹ This polymorphism is greatest in the β chain. As in many other species, ruminants have two hemoglobin types. The embryonal type (HgbE) maintains a dam to in utero O_2 gradient, and the adult type (HgbA) for the ex utero environment. Transition from HgbE to HgbA begins in

utero and may not be complete until months after birth. Like humans, domestic ruminants have a third type of hemoglobin, fetal hemoglobin (HgbF), which replaces HgbE in utero. As gestation progresses to the perinatal period, bovine HgbF is gradually replaced by HgbA. As prenatal RBCs live out their lifespan, HgbF-containing cells give way to HgbA-containing RBCs.

Bovine hemoglobin is a rich source of antimicrobial peptides.³⁰ A peptide isolated by Daoud et al. has antibacterial activity against several organisms including *Escherichia coli* and *Salmonella enteritidis*.⁸ Another group isolated four distinct peptides with similar activity, three which corresponded to the bovine α chain and one to the β chain.³¹ Bovine hemoglobin has also been used extensively as an oxygen-carrying substitute in humans and in small animals.^{5,14}

Erythrocyte Metabolism

The metabolism of domestic animals' erythrocytes was reviewed recently by Harvey.¹⁷ Glucose consumption by bovine RBCs is approximately 0.6 $\mu\text{mol/h/mL}$ RBCs. Most cattle have moderate to high Na^+ and low K^+ RBCs. Some cattle with specific autosomal recessive phenotypes have high intra-erythrocytic K^+ associated with certain blood groups.¹⁷

The ferrous iron of oxyhemoglobin (OxHgb) is constantly being oxidized to the ferric iron of methemoglobin (MetHgb) by intracellular metabolic products.⁹ Erythrocytes normally contain a low concentration of MetHgb that is cyclicly reduced back to OxHgb by the intra-erythrocytic antioxidant enzyme, NADH methemoglobin reductase. When the quantity of MetHgb reaches a certain point, polymerization occurs and the precipitated Heinz body becomes associated with the cell membrane. Accumulation of excessive Heinz bodies may cause both intravascular and extravascular hemolysis.

Erythrocytic 2,3 diphosphoglycerate (2,3 DPG) accumulation modifies the Hgb of some species, but not cattle. Ruminant feti and neonates have greater amounts of erythrocytic 2,3 DPG than adults. However, unlike many other species, 2,3 DPG is not bound as well to ruminant HgbF and HgbA.¹⁵ Bovine HgbF has sufficient inherent oxygen affinity to maintain fetal P_{O_2} .

The copper and zinc metalloenzyme superoxide dismutase was originally identified in cattle.²⁴ Although its value as an in vivo antioxidant is not entirely known, activity of this enzyme is increased in neonatal calves, presumably due to increased oxidative stress associated with parturition.¹¹

Another erythrocyte metalloenzyme of clinical diagnostic significance is selenium-containing glutathione peroxidase (GPX). There is a direct correlation between selenium deficiency and enzyme activity.¹⁸ Although erythrocytic GPX is directly related to white muscle disease, it is inconsistently accompanied by methemoglobinemia. Hemolytic anemia does not frequently occur with selenium deficiency in ruminants. Heinz bodies in selenium-deficient Florida cattle were resolved by selenium treatment.^{17,23}

Differences in hemoglobin types and RBC metabolism of cattle do not appear to make the RBCs of this species any more susceptible to oxidation than those of other species. Cattle have intermediate amounts of the RBC pentose phosphate pathway's limiting enzyme activity, glucose phosphate dehydrogenase.¹⁷ When plants containing large amounts of nitrates or fertilizer nitrates are eaten, microflora reduce the nitrate to nitrite, which when absorbed oxidizes OxHgb to MetHgb without Heinz body formation. Sulfoxide-containing kale and other *Brassica* sp. are converted by rumen microflora to an oxidant that, when absorbed, produces Heinz body hemolytic anemia. Methemoglobin with and without Heinz body formation and hemoglobinuria is associated with consumption of onions and rye grass as well as copper deficiency, toxicosis, and hypophosphatemia.^{6,32}

Erythrocyte Development

During gestation and at birth, the hemoglobin types change and the erythron compartment increases. At birth about 60–90% of the neonatal calf's Hgb is HgbF and 9% of the RBCs are reticulocytes. Fetal calf RBCs are less fragile and larger than adult bovine RBCs. In early gestation, the bovine fetal RBCs have a mean corpuscular volume (MCV) of approximately 95 fL, but by birth it decreases to about 46 fL. From birth to about 8–12 weeks of age, the MCV continues to decrease to approximately 37 fL as HgbF-containing RBCs are replaced by RBCs containing HgbA. The MCV in cattle tends to decline until about 2 years of age.

Veal calves raised solely on a diet of milk or milk replacer have lower RBC values than conventionally raised calves as the result of milk's low iron content.² However, they do not have clinical signs of anemia.

LEUKOCYTES

Although the traditional leukogram is predominantly used to assess leukocytes, cell sorting by immune cell surface markers has improved specificity and sensitivity of cell counting.^{29,34} Specific cell subset profiles, blood and organ distribution, and cytokines are the current subjects of clinical research and development, and may one day supplement or replace the conventional leukogram. Acute phase protein assessment is also frequently used as an additional indicator of inflammation.

Neutrophils

Cattle have a smaller granulocyte bone marrow reserve than many other species, as indicated by a myeloid to erythroid ratio of about 0.5–1.^{3,19} This smaller granulocyte reserve is the reason for neutropenia during the early granulopoietic response to suppurative inflammatory disorders in cattle. Once granulopoiesis is accelerated, a regenerative left shift and neutrophilia develop.¹⁹

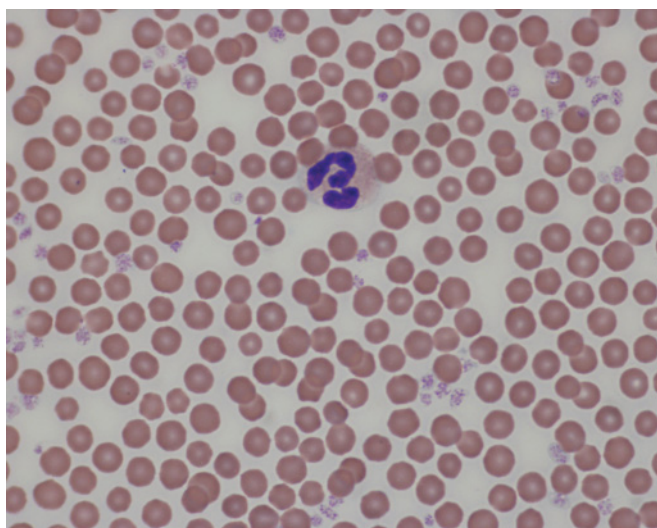


FIGURE 107.1 Segmented neutrophil. Wright's stain; magnification 1000 \times .

The neutrophils of cattle have three types of cytoplasmic granules. This third neutrophil granule is larger than the primary and secondary granules and occupies twice the cytoplasmic space as in sheep and other domestic species, giving the bovine neutrophil its eosinophilic cytoplasm (Fig. 107.1).³ The bovine's large neutrophil granules have more antimicrobial activity than the cytoplasmic granules of nonruminant species.

Cytochemical and immunocytochemical staining reveals a range of enzymes and substrates in ruminant neutrophils.^{1,17,19} Lysozyme, an enzyme common to many species, is not found in the neutrophils of cattle. Immunocytochemical cell membrane antigens serve to add to the specificity of neutrophil characterization³⁴ and proteomic studies assist in further characterizing the molecular profile of these cells.²²

Eosinophils

The ruminant eosinophil has a band to bilobed nucleus which is surrounded by numerous, small, round, intensely stained, red cytoplasmic granules in basophilic cytoplasm (Fig. 107.2).

Parasite-free calves experience a doubling of eosinophil values from 6 months of age to adulthood. As in other animals, parasites and their larvae can produce eosinophilia. Seasonal parasite infestations can be reflected as seasonal eosinophilia. An example is the eosinophilia of subcutaneous migration of ox warble larvae in the spring that produces transient eosinophilia in cattle free of intestinal nematodes.

Basophils

The basophil of cattle is a rare cell about the same size as the eosinophil, with electron-dense, numerous, small, intensely blue-staining granules that sometimes can mask the nucleus (Fig. 107.3). Basophils occur at such

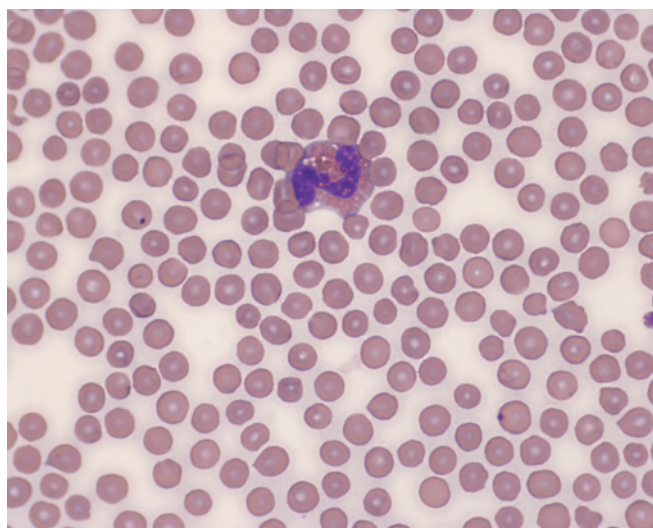


FIGURE 107.2 Bovine eosinophil. Wright's stain; magnification 1000 \times .

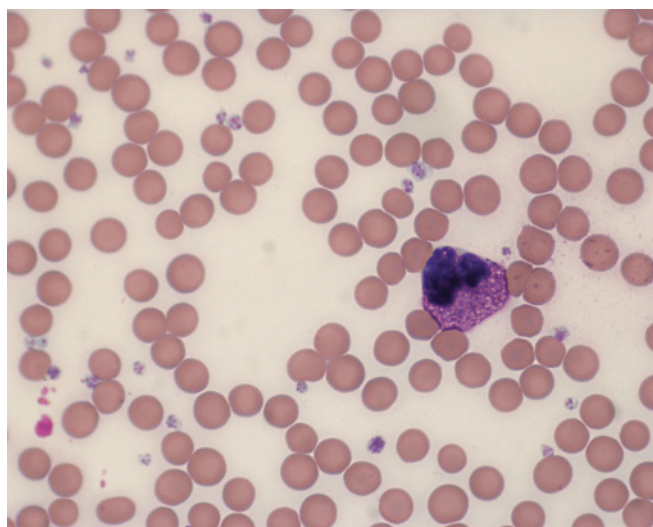


FIGURE 107.3 Bovine basophil. Wright's stain; magnification 1000 \times .

low concentrations that they are not reliably quantified by the 100 differential cell method.

Lymphocytes

As in most species, the ruminant neonate has fewer lymphocytes than granulocytes. In a matter of weeks, the lymphocyte concentration doubles and by 3 months of age they compose 70–80% of the total WBC (Table 107.1). During adulthood, a slow progressive decline in lymphocyte concentration occurs. These age-related fluctuations in lymphocyte concentration exemplify the need for age-related reference intervals.

Bovine lymphocytes are 8–15 μ m in diameter and are described in three sizes. Small lymphocytes have a round, centrally located nucleus with a densely staining

TABLE 107.1 Absolute Leukocyte Values ($\times 10^3/\mu\text{L}$) for Female Jersey Cattle (Mean \pm 1 SD)^a

Age	N	Leukocytes	Segmented Neutrophils	Bands ^b	Lymphocytes	Monocytes	Eosinophils	Basophils ^b
1–6 mo	16	8.8 \pm 2.5	3.0 \pm 1.8	<0.1	4.7 \pm 1.3	0.7 \pm 0.4	0.5 \pm 0.2	<0.1
6–12 mo	10	7.8 \pm 1.8	0.8 \pm 0.5	0	6.3 \pm 1.5	0.6 \pm 0.2	<0.1	0
1–2 yr	14	9.0 \pm 2.5	2.4 \pm 1.4	0	5.9 \pm 1.6	0.4 \pm 0.2	0.5 \pm 0.4	<0.1
2–3 yr	31	9.4 \pm 1.8	2.2 \pm 0.9	<0.1	5.3 \pm 1.2	0.5 \pm 0.2	1.3 \pm 1.0	<0.1
3–4 yr	28	7.7 \pm 1.9	1.9 \pm 1.0	0	4.6 \pm 1.1	0.3 \pm 0.2	0.9 \pm 0.7	<0.1
4–6 yr	29	7.5 \pm 1.1	1.8 \pm 0.7	<0.1	4.0 \pm 0.9	0.5 \pm 0.2	1.2 \pm 0.7	<0.1
>6 yr	21	7.7 \pm 2.5	1.8 \pm 0.9	<0.1	4.3 \pm 2.1	0.4 \pm 0.2	1.3 \pm 0.7	<0.1

^aAdapted from Jain NC. Schalm's Veterinary Hematology, 4th ed. Philadelphia: Lea & Febiger, 1986.

^bMeans only are presented.

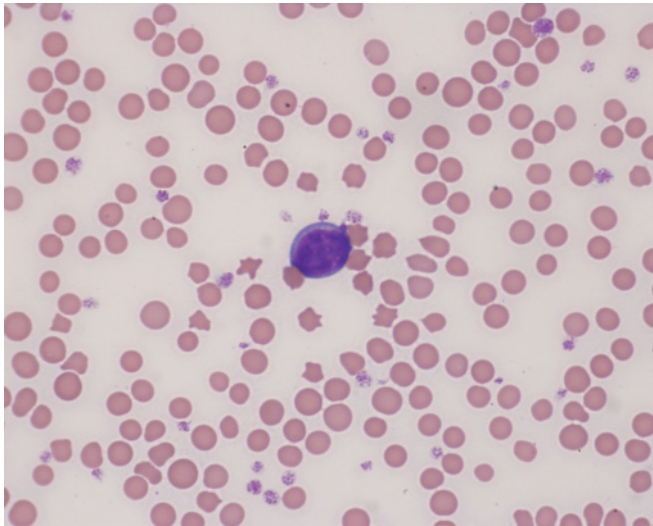


FIGURE 107.4 Small lymphocyte. Wright's stain; magnification 1000 \times .

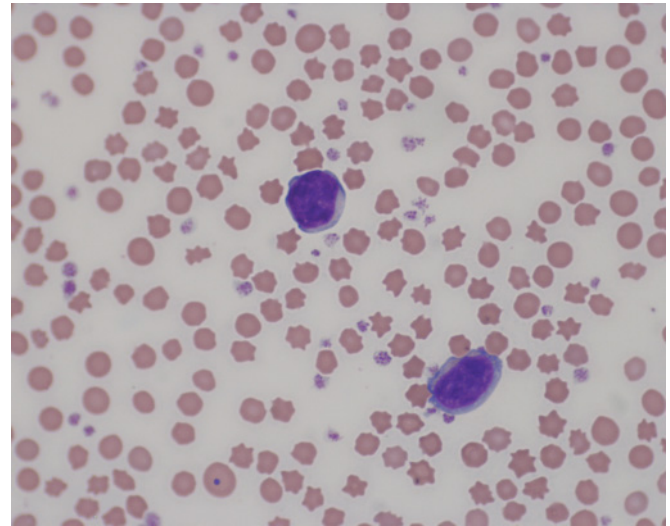


FIGURE 107.5 Small (top left) and medium (bottom right) lymphocytes. Wright's stain; magnification 1000 \times .

chromatin pattern in a small volume of clear to gray-blue cytoplasm (Fig. 107.4). The paucity of cytoplasm and density of the chromatin suggest that the small lymphocyte may be metabolically dormant or a less active cell than the other two forms of lymphocytes. In comparison to small lymphocytes, medium lymphocytes have larger, round to indented-shaped nuclei in which the same amount of chromatin is spread over a greater area, revealing lighter staining parachromatin adjoining areas of condensed chromatin (Fig. 107.5). The cytoplasmic volume of medium lymphocytes is also greater and encircles the central to eccentrically placed nucleus. Large lymphocytes have an even larger, lighter staining nucleus and more cytoplasm than the medium-sized lymphocyte. This large lymphocyte has a round or indented or deeply cleaved centrally placed nucleus. These lymphocytes are frequently confused with monocytes. Like monocytes, the cytoplasm of large lymphocytes stains blue to gray and sometimes contains small clear vacuoles. Variable shapes, sizes, and numbers of magenta cytoplasmic granules are frequently seen in a focal arrangement in the lymphocytes of cattle and may serve to identify the large bovine

lymphocyte. Agranular large lymphocytes and monocytes are ultrastructurally inseparable, and cytochemical and immunocytochemical staining are needed to separate these two cell types. Chromatin centers, nucleolar rings, and nucleoli are seen with low frequency in Wright's-stained normal bovine lymphocytes, and caution must be taken in using these features as evidence of neoplasia for these species.

The subsets of ruminant leukocytes and their cytokines are active subjects of study. Cell sorting technology permits identification of subsets of lymphocytes beyond the T and B cells. Some subsets have a predilection for various anatomic sites, and disease states result in altered blood subset profiles.^{3,7}

Monocytes

The bovine monocyte is a round to convoluted-shaped cell 13–19 μm in diameter. It has a large indented to bilobed to amoeboid nucleus containing a diffuse chromatin pattern in a gray cytoplasm containing fine, small, indistinct, magenta to eosinophilic granules.

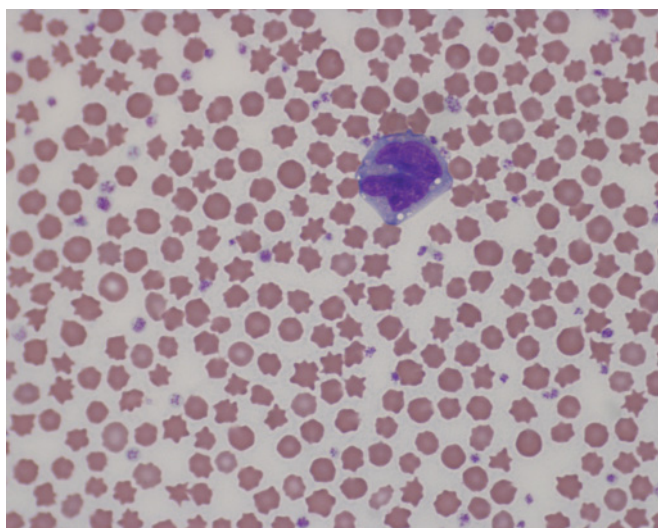


FIGURE 107.6 Bovine monocyte. Wright's stain; magnification 1000 \times .

Vacuoles are frequent and more irregular in shape than those seen in some large lymphocytes (Fig. 107.6).

PLATELETS

Bovine platelets in Wright's-stained peripheral blood are distributed singly or in aggregates, are generally round but can vary in size and shape, and often contain numerous prominent azurophilic granules. Cytoplasmic projections are rare. Normal circulating survival time is thought to be about 10 days. Enlarged platelet forms and pseudopodia are associated with accelerated production of platelets. The ultrastructure of the bovine platelets consists of extensive invaginations of surface membrane.²⁶

GENERAL PHYSIOLOGIC AND ENVIRONMENTAL INFLUENCES

Physiological variables such as recent activity and stress have an impact on hematology values in cattle. Despite the range and sensitivity of technology used, cattle hematology reference intervals are uniformly broad (Table 107.2). Domesticated cattle may have little or no direct physical contact with humans; therefore, when sampling occurs, the animals must be physically restrained and become excited. Reports of reference intervals seldom include consideration of such variables as age, sex, physiologic state, history, form of restraint, ambient temperature, hydration status, BLV status or parasite burdens. It was recently reported that over the past few decades neutrophil counts have increased and lymphocyte counts have decreased compared with those provided in the 1965 edition of *Schalm's Veterinary Hematology*.¹²

Numerous reference values for domestic cattle have been reported and reveal few breed differences. Breed

TABLE 107.2 Hematology Reference Intervals for the Advia 120 from 99 Clinically Healthy Cows, 50% in First Lactation, All Milking 30–150 Days, from 10 Ontario Farms^a

Parameter	Reference Interval	Units
Plasma protein	6.0–8.0	g/dL
Hemoglobin	8.4–12.0	g/dL
Hematocrit	21–30	%
Erythrocytes	4.9–7.5	$\times 10^6/\mu\text{L}$
MCV	36–50	fL
MCH	14–19	pg
MCHC	38–43	g/dL
Reticulocytes	0	$\times 10^3/\mu\text{L}$
RDW	16–20	%
Platelets	160–650	$\times 10^3/\mu\text{L}$
MPV	4.6–7.4	fL
Leukocytes	5.1–13.3	$\times 10^3/\mu\text{L}$
Segmented neutrophils	1.7–6.0	$\times 10^3/\mu\text{L}$
Band neutrophils	0.0–0.2	$\times 10^3/\mu\text{L}$
Lymphocytes	1.8–8.1	$\times 10^3/\mu\text{L}$
Monocytes	0.1–0.7	$\times 10^3/\mu\text{L}$
Eosinophils	0.1–1.2	$\times 10^3/\mu\text{L}$
Basophils	0.0–0.2	$\times 10^3/\mu\text{L}$

^aReference intervals for the Animal Health Laboratory, Laboratory Services Division, University of Guelph, Guelph, Ontario, Canada and were derived from the central 95% of data using parametric or nonparametric statistics using Analyse-It Software. Differential leukocyte counts based on manual counting of 100 cells.

differences have been reported for beef cattle, which have higher RBC values compared to dairy cattle breeds.

Care must be taken to use reference intervals that include similar environmental conditions and seasons as well as physiologic variables observed in the experimental population. Lactating cows have consistently lower white blood cell (WBC), RBC, and plasma protein values than do non-lactating cows.¹⁹ Some reports fail to recognize lactation as a physiologic process and use as their point of reference the general bovine population, including bulls, steers, calves, and non-lactating cows. Bulls have appreciably greater RBC counts than cows.²⁸

Seasonal and environmental changes may influence hematology values. In all species, differing oxygen tensions of altitudes alter the erythron. The greater the altitude, the lower the O₂ tension and the higher the erythron reference intervals. Some western American beef cattle experience two 5,000 ft (1524 m) elevation changes each year that result in a packed cell volume (PCV) change of 10–15%. In the spring they are moved from low winter pastures to high spring and summer pastures and then back down again in the autumn. Seasonal parasite burdens may also alter the complete blood count (CBC).

Aging results in WBC changes (Table 107.1). In the first few weeks of life, neutrophils are the predominant WBCs in calves. By about 2 weeks of age, the lymphocyte becomes the dominant WBC, with a neutrophil:lymphocyte ratio of 0.5 in calves.^{4,27} As bovine adults age, the concentration of neutrophils and lymphocytes decreases but lymphocytes continue to be the predominant cell type.

Pregnancy causes minor changes in RBC and WBC concentrations. At birth, endogenous corticosteroids and the response of excitement and stress result in neutrophilia and lymphopenia.^{19,29} The magnitude of steroid-induced neutrophilia is not as great in cattle as is reported in dogs, cats, and horses.³⁶

BONE MARROW

Extensive studies of the bovine bone marrow in embryos, calves, and adults have been reported. The preferred sites for bone marrow collection in cattle are the sternum and the dorsal ends of the ribs (see Chapter 132). The single most remarkable feature of bovine bone marrow is the low myeloid:erythroid (M:E) ratio of 0.5–0.6 compared with other species. Harvey reports a ratio as low as 0.31.¹⁶ A similar M:E ratio is reported in goats, but in sheep the ratio is about 1. The low bovine bone marrow M:E correlates with the low neutrophil concentration in peripheral blood and the rapid rate at which neutrophils are depleted during inflammation.

PLASMA PROTEINS

The color of bovine plasma ranges from colorless to dark yellow depending on the quantity of dietary plant chromogens being ingested. Summer green feed results in the deepest yellow, and winter dry fodder the lightest.

Precolostral neonatal ruminant plasma proteins are predominately albumin, hepatic origin alpha globulins, with very limited immunoglobulin. In the first day of life, dynamic changes occur in ruminant plasma proteins. Once colostrum is ingested immunoglobulins are quickly absorbed through the rapidly closing gut wall into plasma. Total body water increases, lowering the plasma albumin concentration, while colostral immunoglobulins, and enzymes (gamma glutamyl transferase and alkaline phosphatase) increase to a maximum by 48 hours. Total serum protein concentration in precolostral calves increases from about 4g/dL to as much as 7g/dL. There is an empirical direct relationship between the amount of total plasma protein in the postcolostral calf and frequency of neonatal diseases.³⁸ This relationship is so well established in the veal calf industry that veal calf buyers will frequently only accept calves with greater than 5g/dL of plasma protein.

Plasma fibrinogen and haptoglobin are acute phase hepatic proteins. Fibrinogen has been used as an indicator of inflammatory disorders in cattle for some time, and haptoglobin has been used more recently. Both increase within the first day after onset of inflammation and remain elevated as long as the inflammation is present. As a rule, the greater the magnitude of increase, the greater the magnitude of inflammation. Some individuals regard hyperfibrinogenemia to be as good as or better than the neutrophil count as an indicator of inflammation.

Plasma fibrinogen is low (160 ± 130 mg/dL) to undetectable in neonatal calves. By 3–16 weeks of life it rises to adult values of 300–700 mg/dL.¹⁹ Despite the low baseline value, inflammation can still result in hyperfibrinogenemia of the magnitude seen in adult cattle with inflammatory diseases. Dehydration increases plasma protein concentration, including fibrinogen. Compensation for dehydration in adult cattle can be determined by a ratio of plasma protein to fibrinogen. A ratio of less than 10:1, and more precisely 8:1, is indicative of an absolute increase in fibrinogen. A problem with using the ratio is that hypergammaglobulinemia from chronic inflammation may distort the ratio.

The efficacy of haptoglobin as an indicator of inflammation has been established,^{20,25} but because of greater ease in measurement and reported clinical use, fibrinogen is a more established diagnostic procedure.

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Normal Hematology of Sheep and Goats

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Erythrocytes
Morphology
Hemoglobins
Metabolism
Leukocytes
Neutrophils

Eosinophils
Basophils
Lymphocytes
Monocytes
Platelets
Bone Marrow

Acronyms and Abbreviations

2,3-DPG, 2,3-diphosphoglycerate; EDTA, ethylenediaminetetraacetic acid; EPO, erythropoietin; Hgb, hemoglobin; HgbA, hemoglobin A; HgbC, hemoglobin C; M:E ratio, myeloid:erythroid ratio; PCV, packed cell volume; RBC, red blood cell; seg, segmented neutrophil; WBC, white blood cell.

Domestic and wild sheep and goats are readily excited by the restraint needed for blood sampling. Consequently, in spite of the accuracy of laboratory methods used, the published reference intervals for sheep and goats vary widely (Tables 108.1, 108.2, 108.3 and 108.4).^{2,3,7,15,21–23,34,35}

ERYTHROCYTES

Mammalian erythropoietin (EPO) is genetically well conserved. The efficacious parental use of commercial EPO and the ovine EPO assay has been established.^{14,37,41}

Morphology

Ovine red blood cells (RBCs) are some of the smallest of mammalian RBCs and do not aggregate or deform as readily as RBCs of other species.^{3,25,29,35,36,39} With the exception of the Angora breed, the RBCs of most sheep breeds are discoid in shape. Angora goats frequently have fusiform-shaped RBCs mixed in with discoid RBCs (Fig. 108.1).¹⁶

As in other species, accelerated erythropoiesis in sheep and goats is accompanied by reticulocytosis, macrocytosis, polychromasia, and basophilic stippling

of reticulocytes.¹⁷ Stippling is best demonstrated with freshly prepared, EDTA-preserved, rapidly air dried, Wright's or Giemsa stained RBCs.

Hemoglobins

In addition to increasing the circulating mass of the erythron, adult sheep and goats have a second means of increasing their effective oxygen delivery to tissues at high elevations and in anemias. In response to hypoxia, newly formed RBCs revert to synthesis of hemoglobin C (HgbC) which is normally only produced in immediate postnatal life.^{4,18,40}

Metabolism

The metabolism of domestic animal RBCs has been reviewed by Harvey.¹¹ The ovine RBC glucose consumption rate is 0.7 $\mu\text{mol/h/mL}$ of RBCs in contrast to the caprine RBC consumption rate of 1.9 $\mu\text{mol/h/mL}$ of RBCs.

Plasma in most species contains an appreciably greater Na^+ concentration than K^+ concentration.¹¹ This is in contrast to RBCs where an energy-dependent, membrane pump maintains a high intracellular K^+ concentration and low Na^+ concentration gradient. There is an exception associated with a small proportion of

TABLE 108.1 Normal Blood Values for Sheep^a

	Range	Mean
Erythrocytic series		
Erythrocytes ($\times 10^6/\mu\text{L}$)	9–15	12.0
Hemoglobin (g/dL)	9–15	11.5
PCV (%)	27–45	35
MCV (fL)	28–40	34
MCH (pg)	8–12	10.0
MCHC (%)	31–34	32.5
RBC diameter (μm)	3.2–6.0	4.5
Miscellaneous data		
Plasma proteins (g/dL)	6.0–7.5	
Fibrinogen (mg/dL)	100–500	
Thrombocytes ($\times 10^3$)	800–1,100	500
RBC lifespan (days)	140–150	
Myeloid:erythroid ratio	0.77–1.7	1.1
Leukocytic series		
Total leukocytes ($/\mu\text{L}$)	4,000–8,000	12,000
Neutrophil (band)	Rare	—
Neutrophil (segmented)	700–6,000	2,400
Lymphocyte	2,000–9,000	5,000
Monocyte	0–750	200
Eosinophil	0–1,000	400
Basophil	0–300	50
Percentage distribution		
Neutrophil (band)	Rare	
Neutrophil (segmented)	10–50	30
Lymphocyte	40–55	62
Monocyte	0–6	2.5
Eosinophil	0–10	5.0
Basophil	0–3	0.5

^aFrom Jain NC. Schalm's Veterinary Hematology, 4th ed. Philadelphia: Lea & Febiger, 1986;208–239.

TABLE 108.2 Normal Blood Values for Goats^a

	Range	Mean
Erythrocytic series		
Erythrocytes ($\times 10^6/\mu\text{L}$)	8.0–18.0	13
Hemoglobin (g/dL)	8.0–12.0	10.0
PCV (%)	22–38	28
MCV (fL)	16–25	19.5
MCH (pg)	5.2–8.0	6.5
MCHC (%)	30–36	33
RBC diameter (μm)	2.5–3.9	3.2
Miscellaneous data		
Plasma proteins (g/dL)	6.0–7.5	
Fibrinogen (mg/dL)	100–400	
Thrombocytes ($\times 10^3$)	300–600	450
RBC lifespan (days)	125	
Myeloid:erythroid ratio	0.7	
Leukocytic series		
Total leukocytes ($/\mu\text{L}$)	4,000–1,300	9,000
Neutrophil (band)	Rare	
Neutrophil (segmented)	1,000–7,200	3,200
Lymphocyte	2,000–9,000	5,000
Monocyte	0–550	250
Eosinophil	50–650	450
Basophil	0–120	50
Percentage distribution		
Neutrophil (band)	Rare	
Neutrophil (segmented)	30–48	36.0
Lymphocyte	50–70	56.0
Monocyte	0–4	2.5
Eosinophil	1–8	5.0
Basophil	0–1	0.5

^aFrom Jain NC. Schalm's Veterinary Hematology, 4th ed. Philadelphia: Lea & Febiger, 1986;208–239.

TABLE 108.3 Erythrocyte Parameters in the Normal Goat from Selected Reports Worldwide^{a,b}

Country/Reference	Goat Description	RBC Count ($\times 10^6/\mu\text{L}$)	PCV (%)	Hgb (g/dL)	MCV (fL)	MCH (pg)	MCHC (%)
India ²⁷	0–6 mo old	16.3	27.9	8.0	17.2	—	28.8
	6–12 mo old	13.6	24.3	7.0	17.6	—	28.3
	1–2 yrs old	12.8	22.6	7.4	18.0	—	32.6
	2–3 yrs old	12.6	25.5	7.0	20.5	—	27.3
	3–4 yrs old	10.3	21.9	6.5	22.2	—	29.3
	4–5 yrs old	12.2	24.3	7.0	20.0	—	29.0
	5 yrs and older	12.8	26.0	7.2	21.0	—	27.9
Nigeria ²⁸	0–6 mo old	13.4 \pm 3.3	25.1 \pm 3.4	8.4 \pm 0.9	19.8 \pm 4.8	—	33.9 \pm 3.9
	6–12 mo old	12.9 \pm 2.1	27.0 \pm 4.6	9.1 \pm 1.4	21.2 \pm 3.4	—	33.9 \pm 3.3
	12–24 mo old	11.9 \pm 1.7	26.9 \pm 3.8	8.7 \pm 1.3	22.9 \pm 3.5	—	32.4 \pm 3.2
	2 yrs old	11.8 \pm 2.3	25.9 \pm 4.4	8.5 \pm 1.5	22.4 \pm 4.4	—	32.9 \pm 3.6
	All females	12.2 \pm 2.2	26.1 \pm 4.5	8.5 \pm 1.3	21.8 \pm 3.7	—	33.0 \pm 4.0
	Pregnant females	11.3 \pm 2.0	26.9 \pm 4.0	8.7 \pm 1.6	23.9 \pm 3.6	—	32.3 \pm .9
	All males	12.7 \pm 2.7	25.9 \pm 3.9	8.6 \pm 1.3	21.3 \pm 4.8	—	33.5 \pm 2.9
	All goats	12.3 \pm 2.4	26.1 \pm 4.1	8.6 \pm 1.3	21.8 \pm 4.4	—	33.1 \pm 3.4
United Kingdom ³⁸	Adult males	15.0 \pm 2.4	27.2 \pm 5.2	10.6 \pm 1.6	18.1 \pm 1.7	7.2 \pm 0.8	39.5 \pm 3.6
	Adult wethers	16.3 \pm 2.1	34.8 \pm 3.8	13.1 \pm 1.2	21.4 \pm 0.8	8.1 \pm 0.5	37.7 \pm 2.1
	Adult females	13.9 \pm 2.8	28.9 \pm 5.1	11.4 \pm 1.6	21.1 \pm 3.1	8.4 \pm 1.6	39.6 \pm 4.4
United States ¹⁹	Adults	14.5 \pm 2.9	34.0 \pm 4.9	12.7 \pm 1.5	23.3 \pm 2.1	7.9 \pm 0.4	34.4 \pm 1.5

^aAdapted from Smith MC, Sherman DM. Goat Medicine, 2nd ed. Malvern, PA: Lea & Febiger, 1994.

^bRBC, red blood cell; PCV, packed cell volume; Hgb, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration.

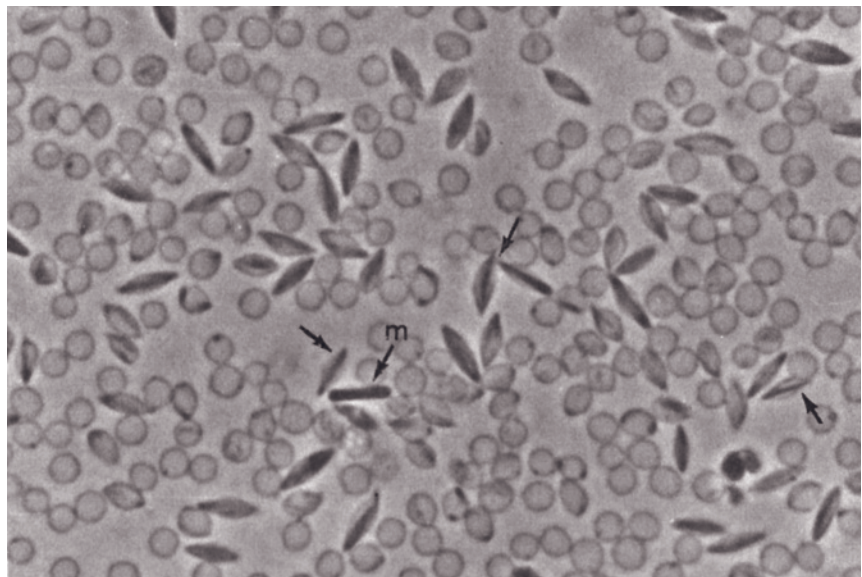
TABLE 108.4 Total Leukocyte Numbers and Differential Counts in Normal Goats Reported Worldwide^a

Country/Reference	Goat Description	WBC count ^b ($\times 10^3/\mu\text{L}$)	Mature Neutrophils (%)	Band Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)	Basophils (%)
Mexico ¹⁰	2 days–7 wks	—	33.7 \pm 12.6	0.9 \pm 0.9	64.1 \pm 13.0	0.8 \pm 0.9	0.5 \pm 0.7	0.0 \pm 0.2
	Adults	—	50.3 \pm 13.7	0.2 \pm 0.4	43.4 \pm 13.9	1.2 \pm 1.1	4.3 \pm 2.1	0.6 \pm 0.8
United Kingdom ¹³	1st day	7.5 \pm 2.9	55.2 \pm 17.9	—	41.3 \pm 14.9	2.0 \pm 1.3	0.7	0.2
	1 wk old	8.9 \pm 4.1	42.9 \pm 11.8	—	52.4 \pm 11.9	2.6 \pm 1.2	0.2	0.5
	1 mo old	9.2 \pm 2.4	32.7 \pm 10.8	—	62.5 \pm 9.4	2.1 \pm 1.7	1.0	1.1
	3 mo old	18.1 \pm 3.8	22.5 \pm 5.8	—	72.6 \pm 11.5	2.0 \pm 3.7	1.1	0.4
	2 yrs old	8.1 \pm 2.5	49.0 \pm 10.7	—	42.3 \pm 10.4	3.1 \pm 2.5	1.9	0.9
	3 yrs old and older	9.7 \pm 2.5	47.7 \pm 12.2	—	48.2 \pm 12.0	2.2 \pm 1.0	1.5	0.2
United States ¹⁹	Adults	13.3 \pm 2.7	43.0 \pm 6.7	—	51.0 \pm 11.4	3.0	2.0	1.0

^aAdapted from Smith MC, Sherman DM. *Goat Medicine*, 2nd ed. Malvern, PA: Lea & Febiger, 1994.

^bWBC, white blood cell.

FIGURE 108.1 Blood film stained with new methylene blue. Partially lysed fusiform RBCs containing a dense bar of polymerized hemoglobin (arrows) can be seen among spheroidal erythrocytic ghost cells. A matchstick form (m) with a dense hemoglobin rod is also present. (Reproduced from Jain NC, Kono CS. Fusiform erythrocytes resembling sickle cells in Angora goats: light and electron microscopic observations. *Res Vet Sci* 1977;22: 169–180, with permission. ©Elsevier)



goats and sheep having specific autosomal recessive blood group phenotypes, in which the RBCs contain a relatively high Na^+ and low K^+ gradient.¹¹

Ruminant fetuses and neonates have greater amounts of RBC 2,3-diphosphoglycerate (2,3-DPG) than do adults. However, unlike some other species, it does not bind to HgbF or HgbA, and an increase in RBC 2,3-DPG does not modify their Hgb oxygen affinity.¹¹

LEUKOCYTES

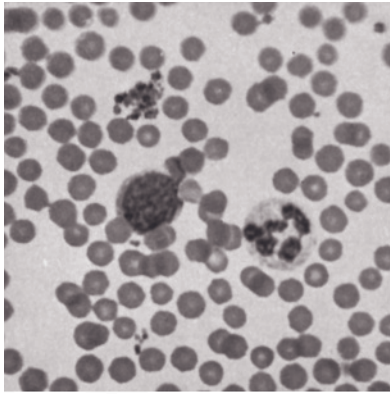
White blood cell (WBC) differential cell counts are useful for monitoring inflammatory states in sheep and goats and are also used in research performed with these species. The intermediate body size and disposition of sheep and goats permit relatively frequent and large blood sampling for research in hematopoiesis, oncology, and immunology.^{6,9,26,30,31}

Ovine and caprine WBC differential cell counts change with age.^{16,20,34} Neutrophils dominate the profile during the first 2 weeks of postnatal life. By 3 weeks of age, lymphocytes are dominant cells with a neutrophil:lymphocyte ratio of 0.5 in lambs and 0.6 in kids. In ewes and does, parturition is accompanied by minor changes in the RBC count and in WBC differential cell counts, such as neutrophilia and lymphopenia.

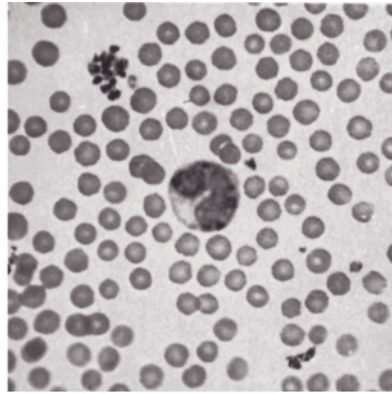
Neutrophils

When Wright's or Giemsa stains are used, the ovine neutrophil cytoplasm has an eosinophilic, granular texture. Neutrophils from sheep and goats contain primary, secondary, and tertiary granules. When compared to other species, tertiary granules are numerous, large and dense^{1,5,12} (Figs. 108.2 and 108.3A; see Chapter 40).

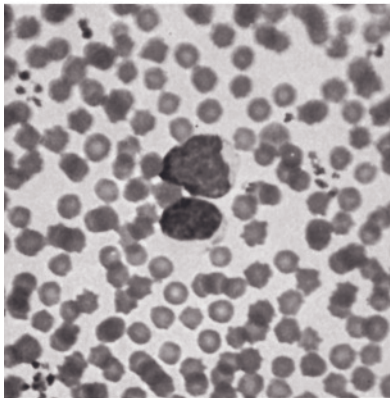
Cytochemical and immunocytochemical staining reveal a range of enzymes and substrates in ruminant



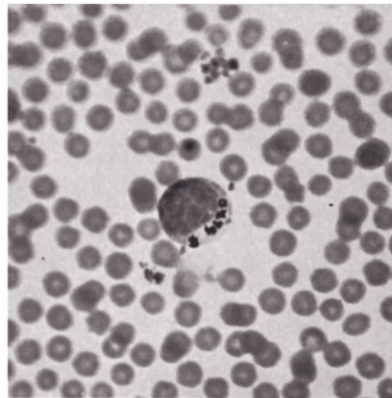
Thrombocytes, lymphocyte, and neutrophil.



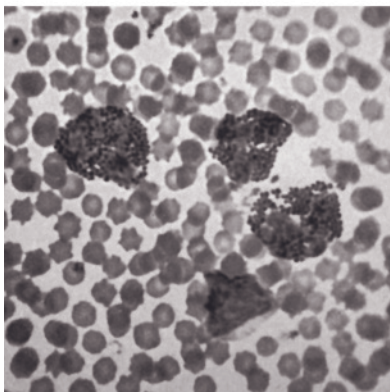
Monocyte and thrombocytes.



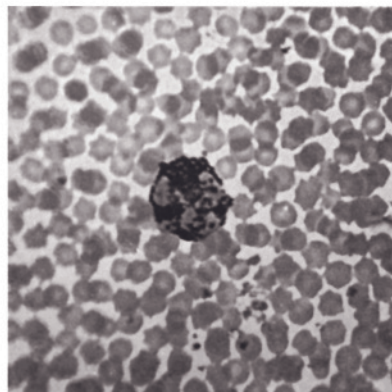
Two lymphocytes.



Lymphocyte with large azurophilic granules.



Three eosinophils and one lymphocyte.



Basophil.

FIGURE 108.2 Ovine blood cells. Original magnification 600×. Top left: Platelets, lymphocyte, and neutrophil. Top right: Monocytes and platelets. Middle left: Two lymphocytes. Middle right: Lymphocytes with large azurophilic granules. Bottom left: Three eosinophils and one lymphocyte. Bottom right: Basophil.

neutrophils (see Chapter 40). Lysozyme is an enzyme common to many species but absent from the cytoplasm of ovine and caprine neutrophils.³²

Eosinophils

The nuclei of most ruminant eosinophils are band or bilobed and surrounded by numerous, intensely red-

stained, small, round, refractile, cytoplasmic granules against the background of a sparse basophilic cytoplasm.¹² Within the ovine eosinophilic granules there are unique dense crystalline structures (Fig 108.2 and 108.3B).^{1,12,16}

The ovine and caprine immunological response to seasonal parasitism is accompanied by eosinophilia.⁸ This seasonal eosinophilia of parasitemia should not be

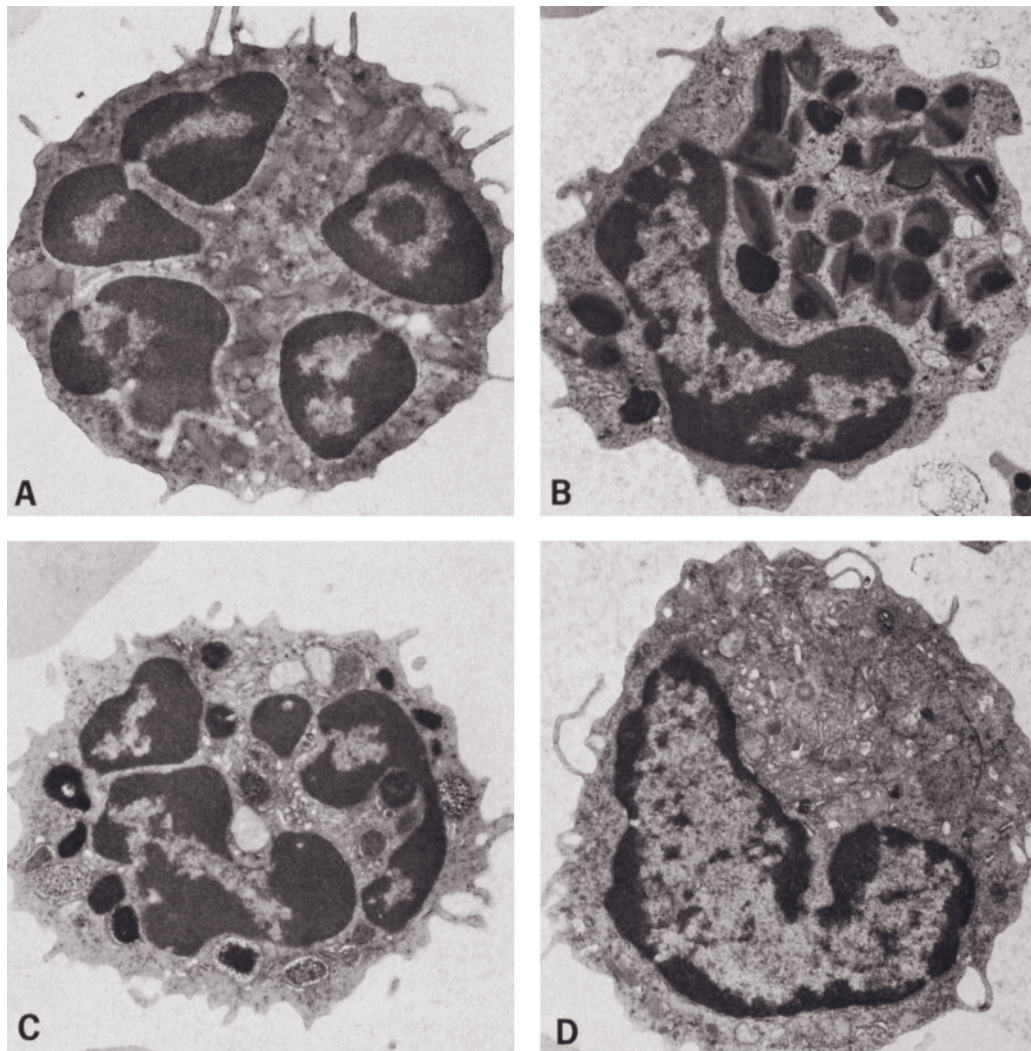


FIGURE 108.3 An ovine neutrophil (A), eosinophil (B), basophil (C), and monocyte (D). (A) The neutrophil has a multilobed nucleus and its cytoplasm is filled with large, electron-dense primary granules and smaller, less-dense specific granules. Few other organelles are seen. Magnification 14,000 \times . (B) The eosinophil is characterized by pleomorphic granules that contain crystalloid rods and membranous whorls. Cellular organelles are sparse. Magnification 12,000 \times . (C) The basophil contains a diverse population of cytoplasmic granules. Maturation of granules proceeds from lightly to densely stippled structures. Magnification 13,000 \times . (D) The cytoplasm of the monocyte has granules, vesicles, and abundant ribosomes. Rough endoplasmic reticulum, mitochondria, and Golgi bodies are prominent. Magnification 13,000 \times . (Courtesy of Dr. Kurt H. Albertine and Dr. Norman C. Staub.)

assumed to be “normal” resulting in inclusion in reference intervals. Investigators concerned with seasonal parasitism eosinophilia have observed it to be earlier and greater in some sheep in a flock. They hypothesize that the magnitude of the eosinophilia is a reflection of the familial immunological resistance to nematode infection.^{30,31}

Basophils

Ovine and caprine basophils are infrequently seen in peripheral blood. When observed they contain numerous, small, electron-dense, intensely basophilic-staining cytoplasmic granules, that may completely mask the nucleus (Fig. 108.3C).^{1,12} Basophils occur so

infrequently in peripheral blood they are not reliably quantified. Marked basophilia is uncommon and may be associated with an eosinophilia.¹⁶

Lymphocytes

Ovine and caprine lymphocytes are small to medium in size and not readily confused with monocytes. They have a sparse blue to gray cytoplasm that frequently contains variably sized and shaped magenta granules of unknown significance (Fig. 108.2).^{1,14,20,23,33}

Lambs and kids begin life with a greater proportion of granulocytes than lymphocytes. Within 3 months of age, lymphocytes represent 70–80% of the total WBC population. Within a few years, the lymphocytes begin

a slow relative decline in numbers.^{13,16,20,23,27} These age-related changes exemplify the need for age-related reference intervals.

Monocytes

Ovine and caprine monocytes are round to convoluted-shaped WBCs with a diameter of 13–19 μm .^{1,12,16} The nucleus is large, indented to bilobed, and contains a diffuse chromatin pattern. The cytoplasm is gray and contains small, indistinct, magenta to eosinophilic granules when stained with Wright's stain. Cytoplasmic vacuoles are common and more irregular in shape than those seen in some large lymphocytes (Figs. 108.2 and 108.3D).

PLATELETS

Wright's-stained blood platelets are distributed singly and in aggregates with azurophilic granules of variable size and shape (Fig. 108.2). Cytoplasmic projections are rare. Normal survival time in the blood is reported to be about 10 days. Giant platelet forms and pseudopodia are associated with recent proliferation from the parent megakaryocytes.²⁴

BONE MARROW

Domestic goats and sheep occur in large commercial herds and flocks as well as in small groups and singles as personal companions. Bone marrow examinations are infrequent in commercial operations, where a clinical examination followed by a necropsy examination is typically more conclusive and cost effective. When concerned with individual animals or small numbers of clinical cases, such as a pet lamb or kid, bone marrow aspirates and biopsies may be desirable. Bone marrow aspirates and biopsies of wild and exotic sheep and goat populations may be informative but capture and restraint precipitate the risk of death.

Sites for obtaining caprine and ovine bone marrow aspirates and core biopsies are the iliac crest and ribs.^{12,33} The single most remarkable difference between non-ruminant and ruminant marrow is the lower myeloid:erythroid (M:E) ratio of hematopoietic cells of the ruminant. Goats have an M:E of 0.5:1 to 0.6:1 and sheep have an M:E of 1:1 (Tables 108.1 and 108.2).^{16,33} Cytologic evidence of accelerated erythropoiesis consists of a left shift of erythropoietic cells and macrophage activity (i.e. nucleophagocytosis, erythrophagocytosis, bilirubin, and iron pigments).^{12,33}

Bone marrow of lambs and kids is predominantly hematopoietic tissue. As they mature, and if adequately fed, marrow fat becomes the dominant feature. As the seasonal availability of food decreases in winter, marrow fat is consumed. Should the poor nutritional state continue, marrow becomes gelatinous in appearance. At necropsy this gelatinous appearance is evidence of a starvation in both domestic and wild sheep

and goats and can be confirmed by low plasma urea nitrogen values.

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Hematology of the Pig

CATHERINE E. THORN

Reference Intervals

Erythrocytes

Leukocytes

Platelets

Bone Marrow

Plasma Proteins

Coagulation

Influence of Age

Influence of Breed and Sex

Influence of Pregnancy, Parturition, and Lactation

Effects of Stress

Effects of Disease

The Fetal Pig

Miniature Swine

Acronyms and Abbreviations

EDTA, ethylenediaminetetraacetic acid; Hgb, hemoglobin; Hp, haptoglobin; MAP, major acute phase protein; RBC, red blood cell; WBC, white blood cell.

Routine hematologic tests are not frequently performed in pigs. The low intrinsic value of individual animals, difficulty in collecting blood, differing husbandry techniques, and wide range reported for many hematologic parameters reduce the utility of the complete blood count for swine. For more detailed information on porcine hematology and hematopoietic organs, reviews have been previously published.^{22,33,55,60} An extensive collection of photographs of porcine blood and bone marrow cells has been published.⁵⁹ Ethylenediaminetetraacetic acid (EDTA) anticoagulant and a Romanowsky-type stain are preferred for optimal cytological evaluation. Because porcine red blood cells (RBCs) are relatively fragile, excess turbulence or improper handling of the sample frequently results in hemolysis. Routine hemogram values, except the differential cell count, are stable at 20°C or 4°C for up to 36 hours, whereas the white blood cell (WBC) differential cell count becomes less reliable within 12 hours after collection.²⁰ Automated counting of leukocyte subsets⁷⁵ and reticulocytes²⁴ has been reported.

Blood collection in pigs can be difficult due to poor accessibility of veins as well as variables associated with animal cooperation. Adequate blood volume can be obtained from the external jugular or anterior vena cava. Other sites include the ear (auricular) vein, tail vein, and subcutaneous abdominal vein, also known as the milk vein.³¹ Intraosseous needle placement has also been reported.²⁸

REFERENCE INTERVALS

Many reference intervals for porcine blood have been published.^{8,18,23,59,60} Although most published intervals are comparable, the ranges for most elements of porcine blood are quite wide (Table 109.1). Variability may be due to sex, breed, growth rate, diet, age, stage of gestation or lactation, feeding method, management practices, or season.^{8,14,78} Interpretation of porcine hematology data requires consideration of these factors.

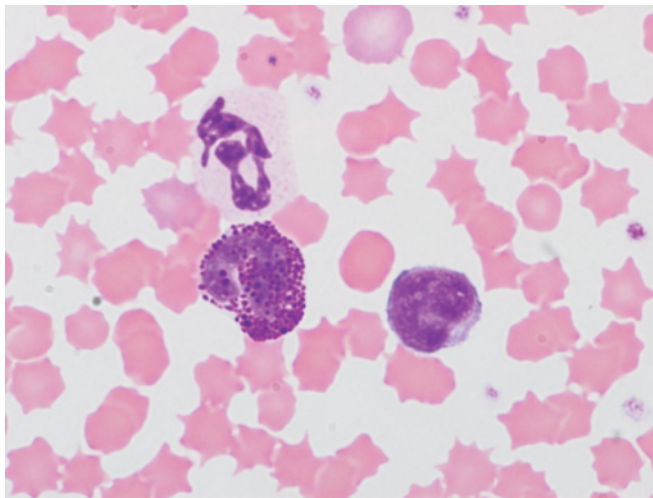
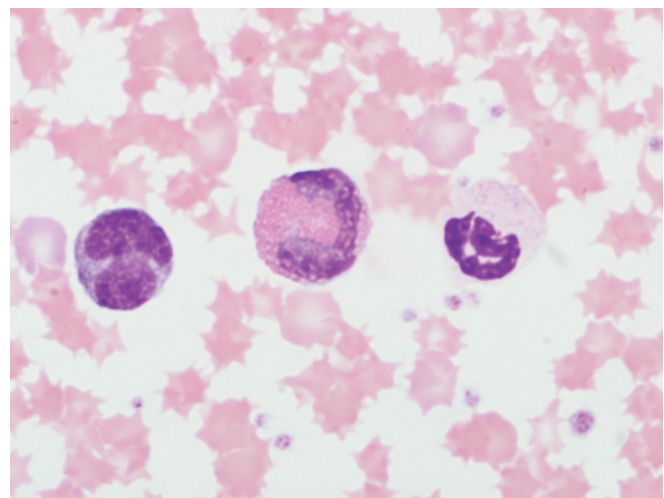
ERYTHROCYTES

Porcine RBCs have an average diameter of 6.0 μm. Artifacts such as crenation are frequently encountered, and the cells tend to form rouleaux in health (Fig. 109.1). Central pallor is not visible in every cell. Anisocytosis is seen in adult pigs but is more prominent in younger pigs. Blood from young pigs contains many large polychromatic RBCs, nucleated RBCs and Howell-Jolly bodies.

The pig RBC is highly susceptible to hemolysis by hypotonic saline. Porcine RBCs have a bimodal pattern, with RBCs from adult and fetal pigs being more resistant to lysis than RBCs from weaned pigs. Osmotic resistance is temperature-, pH- and time-dependent,^{49,50} but not influenced by sex or breed.³⁹ The sedimentation rate of pig RBCs is faster than that of other domestic animals^{55,77} and is subject to daily fluctuations.³²

TABLE 109.1 Reference intervals for the Domestic Pig^a

Erythrocytic Series			Leukocytic Series		
	Range	Average		Range	Average
Erythrocytes ($\times 10^6/\mu\text{L}$)	5.0–8.0	6.5	Leukocytes ($/\mu\text{L}$)	11,000–22,000	16,000
Hemoglobin (g/dL)	10.0–16.0	13.0	Percentage distribution		
PCV (%)	32–50	42.0	Neutrophil (band)	0–4	1.0
MCV (fL)	50–68	60	Neutrophil (mature)	28–47	37.0
MCH (pg)	17.0–21	19.0	Lymphocyte	39–62	53.0
MCHC (%)	30.0–34.0	32.0	Monocyte	2–10	5.0
Reticulocytes (%)	0.0–1.0	0.4	Eosinophil	0.5–11	3.5
ESR (mm in 1 h)	Variable		Basophil	0–2	0.5
RBC diameter (μm)	4.0–8.0	6.0	Other Data		
RBC lifespan (days)	86 \pm 11.5		Thrombocytes ($\times 10^5/\mu\text{L}$)	5.2 \pm 1.95	
Resistance to hypotonic saline (%)			Icterus index (units)	<5	
Min.		0.70	Plasma protein (g/dL)	6.0–8.0	
Max.		0.45	Fibrinogen (g/dL)	0.1–0.5	
Myeloid:erythroid ratio	1.77 \pm 0.52:1 (Lahey et al. 1952)				

^aSee reference 69.**FIGURE 109.1** Erythrocytes, neutrophil (top left), basophil (center), large lymphocyte (bottom right) and platelets. (Courtesy of Dr. Charles Brockus.)**FIGURE 109.2** Monocyte (left), eosinophil (center), neutrophil (right) and platelets. (Courtesy of Dr. Charles Brockus.)

LEUKOCYTES

The mature neutrophil is 12–15 μm in diameter. It has an irregular nuclear membrane and moderately coarse chromatin with well defined lobes (Figs. 109.1 and 109.2). The cytoplasm stains pale pink or blue and contains a few pink granules. Band neutrophils have a U-shaped nucleus but are otherwise similar to the mature form (Fig. 109.3). Band cells may be present in healthy pigs. Metamyelocytes have less mature chromatin patterns, and nuclear shape may vary from kidney-bean-shaped to ring forms with no lobation. The

cytoplasm is pale blue. Eosinophil nuclei are poorly segmented and may appear immature. The cytoplasmic granules are round to oval, stain a pale orange color and tend to fill the cytoplasm (Fig. 109.2). The basophil nucleus stains lavender and has a smooth chromatin pattern (Fig. 109.1). The cytoplasmic granules of the basophil are coccoid to dumbbell-shaped and stain similarly to or more intensely than the nucleus.

Small lymphocytes are 7–10 μm in diameter (Fig. 109.4), have a round to oval nucleus with a condensed chromatin pattern, and a small rim of pale blue cytoplasm. Large lymphocytes are 11–15 μm in diameter

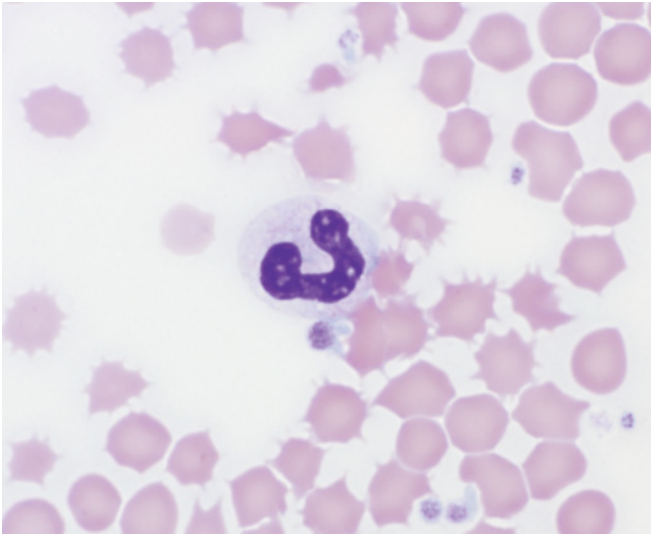


FIGURE 109.3 Band neutrophil. (Courtesy of Dr. Charles Brockus.)

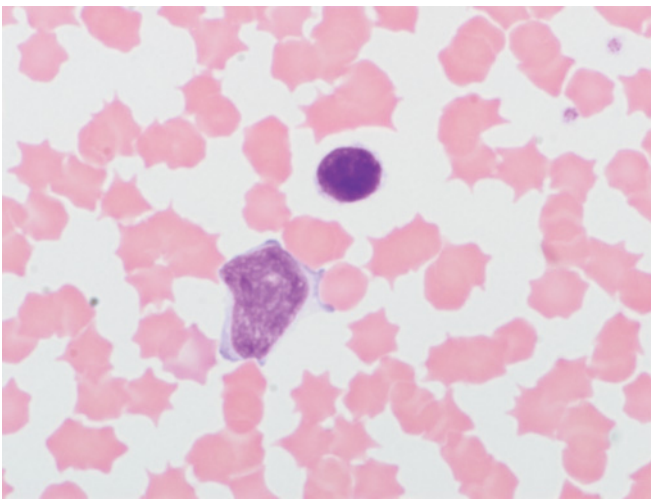


FIGURE 109.4 Large lymphocyte (bottom) and small lymphocyte (top). (Courtesy of Dr. Charles Brockus.)

(Figs. 109.1 and 109.4). The chromatin pattern is slightly coarse and does not stain as intensely as the small lymphocyte. The cytoplasm stains pale blue. Large lymphocytes may contain low numbers of round to oblong azurophilic granules usually located at the margin of the cell (Fig. 109.5). Many circulating lymphocytes are referred to as “null cells” based on lack of expression of cell surface antigens. Some T cells possess both CD4 and CD8 surface markers. The CD4:CD8 ratio is 0.6.⁵⁷

Monocytes are 14–18 μ m in diameter and have a convoluted nucleus with lacy chromatin and focal condensed areas (Fig. 109.6). The abundant blue-gray cytoplasm may contain granules or vacuoles. Some monocytes may be difficult to distinguish from a large lymphocyte or immature neutrophil.³²

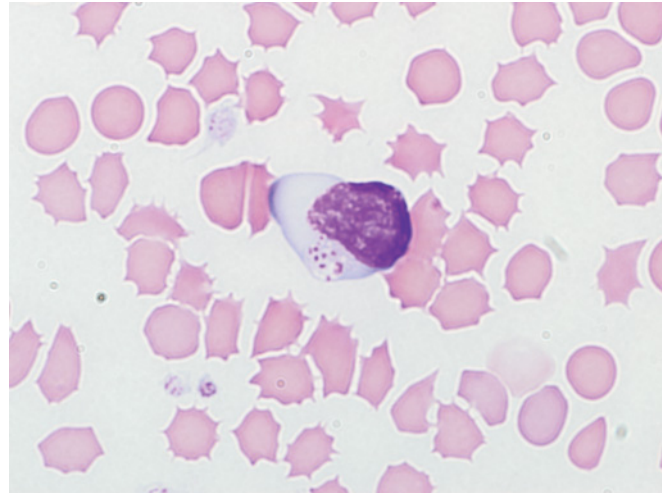


FIGURE 109.5 Granular lymphocyte. (Courtesy of Dr. Charles Brockus.)

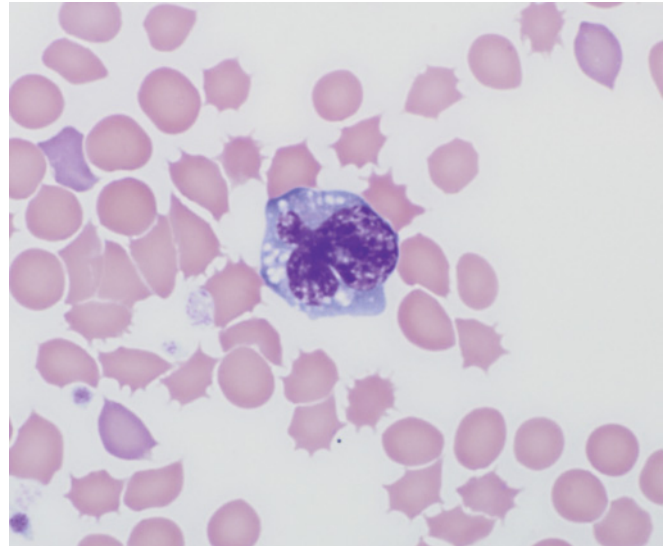


FIGURE 109.6 Monocyte. (Courtesy of Dr. Charles Brockus.)

PLATELETS

Porcine platelets are morphologically similar to those of other domestic species (Figs. 109.1 and 109.2). They are variable in shape and are generally small (i.e. 1–3 μ m in diameter) with a mean volume of 6.9–8.9 fL.¹⁸ Platelets are anucleate and have deeply staining purple cytoplasmic granules. Platelets are frequently arranged in variably sized clumps.

BONE MARROW

Bone marrow samples are rarely evaluated in pigs. Myeloid to erythroid ratios of 1.77:1 to 2:1 have been reported.^{33,59}

PLASMA PROTEINS

Plasma protein concentration in adult pigs is about 7.0–8.0 g/dL.⁴² Fibrinogen concentration greater than 1.0 g/dL in adult pigs on an indication of inflammation.

The measurement of other serum acute phase proteins is increasingly being utilized for identification of acute inflammation as well as indicators of immunological stress and subclinical infection. Haptoglobin (Hp) and C-reactive protein have been identified as major acute phase proteins in the pig.^{11,36} Major acute phase protein (MAP) has also been reported in pigs.³⁶ Measurement of serum Hp concentration aids in identifying subclinical infection with various respiratory agents^{21,29,30} as well as providing a marker for production parameters such as growth rate¹⁷, general hygiene³⁷ and injury.⁵³ Serum reference intervals for pigs from commercial farms have been reported for pig-MAP and Hp, and were found to vary with gender and age. Pig-MAP serum concentrations were lower in sows than in boars (mean values 0.81 vs. 1.23 mg/mL) while the opposite was found for Hp (1.47 vs. 0.94 mg/mL). Pig-MAP generally decreases with age while mean Hp concentration increases.⁵⁴

COAGULATION

Coagulation of pig blood not collected into anticoagulant is rapid.²² Coagulation parameters, tests for bleeding time and platelet function in pigs have been described but are seldom used.^{1,4,32}

INFLUENCE OF AGE

Blood values for newborn and young piglets have been published.^{13,33,48,59} Table 109.2 depicts the changes in the RBC and WBC parameters of piglets farrowed and kept on concrete for 10 days, then transferred outside to soil.

Many RBC changes take place after birth. Within a few days of age, RBC number and hemoglobin (Hgb) concentration drop 30–38% due to expansion of plasma volume.⁴³ Cell size increases soon after birth, decreases to its smallest size at 2–6 months of age, and then increases again to adult size. In suckling pigs, reticulocyte counts of 3–8% and nucleated RBC counts of 5% are common. These decrease as pigs mature. Polychromasia, Howell-Jolly bodies, crenation, rouleaux, and poikilocytes are frequently seen in the blood of young pigs. RBC counts and Hgb concentration increase to reach adult levels at about 5 months of age.

The WBC count is high at birth. Total WBC count decreases shortly after birth, then increases at about the fifth week of life. Actual cell numbers are quite variable. At birth, neutrophils represent about 65–70% of the leukocytes and lymphocytes account for about 20%. In the first week, neutrophils and lymphocytes are present in equal numbers; by 10 days of age, lymphocytes outnumber neutrophils. The ingestion of colostrum and

age at weaning affect RBC and WBC numbers, and cell dynamics.^{26,72} By 6 months, the neutrophil:lymphocyte ratio is about 1:2.

The proliferation of available monoclonal antibodies to pig T-cell and B-cells^{2,58} has revealed the dynamics of pig peripheral blood lymphocytes throughout varying stages of growth. Proportions of T-cell and B-cell subsets have been monitored during the neonatal period³ and in prefattening pigs.⁶⁸ Production performance as measured by average daily gain, carcass measurements, feed intake, feed conversion and the ability to predict growth, has been associated with the presence or absence of certain lymphoid phenotypes.²⁷

Platelet numbers appear to be age dependent, but reported trends are inconsistent.^{1,48} The concentration of plasma protein before and after the ingestion of colostrum is about 2.2 g/dL, and 5.5 g/dL respectively. Plasma proteins slowly increase during the first year of life to 7–8 g/dL.⁴² In healthy pigs 2–3 months of age, plasma fibrinogen concentration ranges from 0.2 to 0.4 g/dL.

The rapid growth rate of young pigs places a large demand on iron requirements. Milk alone cannot meet this requirement. Unless a supplemental source of iron is provided, pigs develop a severe microcytic, normocytic to hypochromic anemia. Serum ferritin and total iron binding capacity become reduced.⁶³ Early contact with soil³⁵ or intramuscular injection of iron dextran is recommended.²² Spontaneous recovery occurs about the fifth week of life when the pigs start to derive nutrients from other sources. The effect of low iron on WBC counts is variable;^{25,66} however, iron deficient piglets have been shown to have decreased immunocompetence.⁶⁷

INFLUENCE OF BREED AND SEX

There is no consistent significant influence of gender on hematologic parameters. Observed differences were not considered biologically important.^{12,14,40,46} Breed differences in values have been reported.^{12,19,34,78}

INFLUENCE OF PREGNANCY, PARTURITION, AND LACTATION

Hematologic changes occur with pregnancy, parturition, and lactation (Table 109.3). Approximately 2 weeks before parturition, RBC parameters in sows decrease and continue to do so until the end of lactation. Reticulocytes may be seen during this period. Total WBC number decreases during gestation, and anemia during pregnancy in sows has been documented.^{9,44} Neutrophilia with a left shift and lymphopenia frequently occur at parturition. Within 24 hours of farrowing the neutrophil:lymphocyte ratio reverses.⁴⁵

The influences of treatment with recombinant porcine somatotrophin,⁶⁴ growth hormone-releasing factor, and thyrotropin-releasing factor on sow blood during lactation have been reported.⁹

TABLE 109.2 Influence of Age and Husbandry on Hematologic Values of Young Duroc-Jersey Pigs. Values are Ranges and Means for a Single Litter of 5 Males and 4 Females. Pigs Were Kept on Concrete Until 10 Days of Age and Then Placed on Soil^{a,b}

Age (days)	Value	Weight (kg)	RBC ($\times 10^6/\mu\text{L}$)	Hgb (g/dL)	PCV (%)	MCV (fL)	MCHC (%)	MCH (pg)	Retic. (%)	Nuc. RBC/100WBC	Sed. Rate (1 h)	WBC ($\times 10^3/\mu\text{L}$) ^c	Differential Leukocyte Count (%)					
													Band	Neutrophil	Lymphocyte	Monocyte	Eosinophil	Basophil
1	Min.	0.77	4.3	8.4	27.0	57	28.9	18.0	4.5	0.5	0	7.6	1.0	64.5	16.0	0.5	0	0
	Max.	1.50	6.4	12.3	42.5	71	31.3	21.0	10.0	4.0	4	15.3	7.0	75.5	31.0	7.5	2.0	1.0
	Ave.	1.09	5.3	10.5	35	67	30.5	20	6.7	2	2	11.5	3.6	71	20	4.7	0.9	0.2
3	Min.	1.09	3.3	7.8	26.5	70	29.1	21.0	6.9	7	2	6.3	1.0	38.0	23.5	6.0	0	0
	Max.	1.81	5.2	11.0	36.5	81	30.3	24.0	16.6	57	12	13.4	5.5	61.5	54.0	9.5	1.5	0
	Ave.	1.45	4.5	9.8	33	73	29.5	22	12.0	17	5	9.4	3.3	51	37.6	6.8	0.8	0
6	Min.	1.59	3.4	6.4	22.0	60	26.4	17.0	4.5	5	12	7.4	1.0	33.0	32.5	2.0	0	0
	Max.	2.27	4.7	9.4	31.0	74	30.9	23.0	13.0	54	33	10.5	3.3	60.5	55	10.5	1.0	0
	Ave.	2.04	4.0	8.0	26.7	67	29.1	20	7.7	14	22.6	8.2	2	45.4	45.3	4.9	0.3	0
10	Min.	2.36	2.1	4.2	15.0	62	29.0	19.0	6.0	3	1	5.6	0	8.0	36.5	1.0	0	0
	Max.	3.22	4.3	8.7	20.0	78	31.0	24.0	12.0	30	35	19.1	2.0	51.0	82.0	10.0	0.5	0.5
	Ave.	2.90	3.5	7.0	24	68	29.6	20	10	11	12	10.9	1	27	64	7	0.1	0.05
20	Min.	3.85	4.4	9.0	35.5	70	26.0	19.0	9.0	1	0	6.2	0	13.5	55.0	2.0	0	0
	Max.	5.22	5.3	11.2	40.5	82	29.0	23.0	13.0	25	1	10.5	3.5	39.5	82.0	7.0	2.0	0.5
	Ave.	4.76	4.9	10.2	37	76	27.6	21	10.6	11.5	0	7.7	1.4	25.7	66.8	4.3	0.8	0.05
36	Min.	—	5.9	11.3	37.0	62	28.0	18.8	1.6	0	0	12.7	0	28.0	40.0	3.0	3.5	0
	Max.	—	6.8	13.3	44.0	68	32.0	20.0	6.8	1	2	20.9	5.0	43.0	68.0	10.5	14.0	1.5
	Ave.	—	6.2	12.1	39.7	64	30.5	19.4	3.0	0.5	0.5	16.3	1.8	33	52	6	7	0.5

^aThese data were developed in cooperation with Dr. Otto Straub.

^bSee Thorn CE. Normal hematology of the pig. In: Feldman BF, Zinkl JG, Jain NC, eds., Schalm's Veterinary Hematology, 5th ed. Philadelphia, Lippincott Williams and Wilkins. 2000;1089–1095.

^cCorrected for nucleated RBCs.

TABLE 109.3 Influence of Age, Sex, Castration, Pregnancy, and Parturition on Hematologic Variables of Duroc-Jersey Swine^{a,b}

Classification	No.	Value	RBC ($\times 10^6/\mu\text{L}$)	Hb (g/dL)	PCV (%)	MCV (fL)	MCHC (%)	Sed. Rate (1 h)	WBC ($\times 10^3/\mu\text{L}$)
Both sexes 3½–4 mo	10	Min.	6.4	11.5	38	53	28	0	18.9
		Max.	8.0	13.3	44	61	31	6	33.8
		Ave.	7.1	12.0	40	57	30	2.6	26.9
Castrated males 3–6 mo	16	Min.	6.0	9.8	31	54	28	0	11.1
		Max.	8.0	13.0	44	68	32	25	28.3
		Ave.	7.0	11.7	39	59	30	5	19.5
Males 6–12 mo	9	Min.	6.3	11.5	37	55	29.5	1	13.4
		Max.	8.6	13.5	44	68	33	14	25.3
		Ave.	7.0	12.4	41	59	31	5	18.9
Males 1 year & older	8	Min.	5.8	12.8	41	62	30	0.5	10.0
		Max.	7.5	15.3	50	72	33	31	17.4
		Ave.	6.7	14.1	45	66	31	13	13.3
Females 6–12 mo; not pregnant	10	Min.	5.4	10.4	36	53	30	5	14.5
		Max.	7.9	13.8	46	67	34	27	21.6
		Ave.	7.0	12.9	41	59	32	15	15.3
Females 1 yr and over; not pregnant	9	Min.	4.7	9.6	31	56	30	24	11.6
		Max.	7.7	14.3	48	69	33	55	21.0
		Ave.	6.0	12.1	38	64	31	26	16.4
Females 1 yr & over; pregnant 3–8 weeks	20	Min.	5.6	11.5	37	58	30	1	11.3
		Max.	8.0	14.7	48	68	32	30	22.3
		Ave.	6.4	13.3	43	63	31	7	16.3
Females 1 yr & over; pregnant 2½–3½ months	38	Min.	5.1	11.2	35	59	29	3	9.8
		Max.	8.0	15.3	50	69	33	53	20.9
		Ave.	6.4	12.8	42	65	31	20	14.4
Females 2 weeks or less before parturition	14	Min.	4.9	11.0	34	63	30	0	11.5
		Max.	6.3	14.5	46	75	33	47	21.9
		Ave.	5.7	12.6	40	70	31.6	21	15.6
Females 1–6 h postpartum	5	Min.	4.9	11.2	35	57	31	21	15.1
		Max.	6.5	12.8	42	73	32	45	19.2
		Ave.	5.7	12.1	38	66	31.8	33	17.3
Females 10–24 h postpartum	8	Min.	4.5	10.0	30	57	31	10	7.0
		Max.	7.3	14.5	46	70	33	54	17.2
		Ave.	5.8	12.0	37	63	32	47	10.3
Females 2–10 days postpartum	13	Min.	4.5	9.8	30	59	31	0	7.8
		Max.	6.9	15.1	47	71	34	28	21.0
		Ave.	5.5	12.7	39	66	32	12	15.0
Females 15–49 days postpartum	10	Min.	2.4	5.1	15	61	29	3	8.8
		Max.	6.0	12.3	42	79	35	55	24.4
		Ave.	4.9	10.4	32	66	32	35	18.7

^aThese data were developed in cooperation with Dr. Otto Straub.

^bSee Thorn CE. Normal hematology of the pig. In: Feldman BF, Zinkl JG, Jain NC, eds., Schalm's Veterinary Hematology, 5th ed. Philadelphia, Lippincott Williams and Wilkins, 2000;1089–1095.

EFFECTS OF STRESS

In swine, sampling stress is the largest source of hematologic variation.⁷ The stress response develops within 2 minutes, rapidly affecting the leukogram.⁸ The total WBC and neutrophil count can increase by two- to three-fold, resulting in a stress leukogram with significant elevations also seen in Hgb concentration, hematocrit and RBC sedimentation rate.¹⁵ Long- and short-term treatment with dexamethasone in weanling piglets causes a significant decrease in the percentage of peripheral blood CD8+ T cells.³⁸

The effect of sedation on hematological values is unpredictable; therefore, sedation is not recommended for blood collection.¹⁸

EFFECTS OF DISEASE

Hematologic changes associated with various inflammatory processes in swine have been examined.^{46,62,73} Increased RBC sedimentation rates as well as decreased hematocrit and hemoglobin concentration have been reported.¹⁸ Evaluation of a blood smear with a differen-

TABLE 109.3 *continued*

Classification	No.	Value	Differential Leukocyte Count (%)							
			Myelocyte	Metamyelocyte	Band	Neutrophil	Lymphocyte	Monocyte	Eosinophil	Basophil
Both sexes 3½–4 mo	10	Min.	—	—	1.0	17	46	1.0	0.5	0.0
		Max.	—	—	3.0	42	77	8.0	8.5	1.5
		Ave.	0.0	0.0	2.0	27	63	5.0	2.5	0.5
Castrated males 3–6 mo	16	Min.	—	—	0.5	17	44	2.0	0.0	0.0
		Max.	—	—	4.5	45	73	10.0	10.0	1.0
		Ave.	0.0	0.0	2.0	29	60	6.0	2.5	0.5
Males 6–12 mo	9	Min.	—	—	0.0	30	51	4.0	0.5	0.0
		Max.	—	—	2.5	41	61	9.0	4.5	1.0
		Ave.	0.0	0.0	1.5	33	57	6.0	2.2	0.3
Males 1 year & older	8	Min.	—	—	0.0	11	36	5.0	2.0	0.0
		Max.	—	—	2.0	49	76	12.0	5.5	3.0
		Ave.	0.0	0.0	0.6	32	55	8.0	3.5	0.9
Females 6–12 mo; not pregnant	10	Min.	—	—	0.5	19	53	3.5	0.0	0.0
		Max.	—	—	3.5	37	67	10.5	5.5	2.0
		Ave.	0.0	0.0	1.2	33	57	5.5	2.7	0.6
Females 1 yr and over; not pregnant	9	Min.	—	—	0.0	28	38	0.0	0.5	0.0
		Max.	—	—	2.0	42	61	9.0	10.0	1.5
		Ave.	0.0	0.0	0.7	36	54	5.0	4.0	0.3
Females 1 yr & over; pregnant 3–8 weeks	20	Min.	—	—	0.0	31	39	2.5	1.0	0.0
		Max.	—	—	2.5	48	61	11.0	12.0	2.0
		Ave.	0.0	0.0	1.0	37	51	6.0	4.0	1.0
Females 1 yr & over; pregnant 2½–3½ months	38	Min.	—	0.0	0.0	23	30	0.5	0.0	0.0
		Max.	—	0.5	4.5	58	68	12.0	9.0	2.0
		Ave.	0.0	0.1	1.1	35	55	5.0	3.0	0.8
Females 2 weeks or less before parturition	14	Min.	—	—	0.0	25	34	1.5	0.0	0.0
		Max.	—	—	3.5	55	57	9.0	2.5	1.5
		Ave.	0.0	0.0	1.7	39	52	6.0	0.7	0.6
Females 1–6 h postpartum	5	Min.	—	0.0	0.0	43	17	0.5	0.5	0.0
		Max.	—	1.0	5.5	67	48	9.0	11.5	0.0
		Ave.	0.0	0.2	2.8	52	33	5.0	7.0	0.0
Females 10–24 h postpartum	8	Min.	0.0	0.0	0.0	8	34	2.5	1.0	0.0
		Max.	4.0	6.0	42.0	45	72	11.0	6.0	1.5
		Ave.	0.5	1.8	10.0	30	46	7.0	4.0	0.7
Females 2–10 days postpartum	13	Min.	—	—	0.0	4	29	2.5	0.5	0.0
		Max.	—	—	7.5	58	57	30.0	9.5	3.0
		Ave.	0.0	0.0	3.0	43	40	9.0	4.0	1.0
Females 15–49 days postpartum	10	Min.	—	0.0	0.5	36	31	2.0	2.0	0.0
		Max.	—	3.0	14.0	59	52	11.5	10.0	3.5
		Ave.	0.0	0.6	4.0	46	37	6.0	5.0	1.4

tial cell count may be a better indicator of an inflammatory process than total WBC counts.²²

THE FETAL PIG

A number of studies have reported the hematologic characteristics of fetal pigs.^{6,9,10,74} Nucleated RBCs are present in the fetal circulation at 30 days' gestation. Red blood cell number and Hgb concentration increase with age as nucleated RBCs decrease. Red blood cell size decreases during gestation.^{9,74} Reticulocyte percent is maximal (6.5%) at mid gestation, but decreases to 1% by birth. Total WBC number increases throughout

embryonic life consisting mostly of neutrophils. Serum protein concentration is 2–3 g/dL throughout gestation, with no sex or breed differences reported.

MINIATURE SWINE

Miniature pigs are an important animal model in biomedical research^{51,71,76} and are also popular as pets.^{5,61} Hematologic parameters of many breeds of miniature pigs have been characterized, with similar characteristics and cell morphology as larger domestic swine.^{41,52,56,70} There was no effect on RBC or WBC parameters with

regard to sex,^{47,52,56} but breed differences have been reported.¹⁶ Age-related changes in miniature pigs are comparable to those in domestic swine.^{16,47,52} Adult values are attained at about 9 months of age. Blood collection in these smaller pigs can be obtained easily and safely via the subcutaneous abdominal vein. Restraint is minimal and most of the pigs tolerate it well.⁶⁵

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Hematology of Laboratory Animals

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Acronyms and Abbreviations

Hct, hematocrit; Hgb, hemoglobin; IL, interleukin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; M:E, myeloid to erythroid; NHP, non-human primate; PCT, platelet crit; PCV, packed cell volume; PDW, platelet distribution width; RBC, red blood cell; RDW, red cell distribution width; WBC, white blood cell.

HEMATOLOGY OF LABORATORY RODENTS: MOUSE (*MUS MUSCULUS*) AND RAT (*RATTUS NORVEGICUS*)

ANNE PROVENCHER BOLLIGER and NANCY E. EVERDS

The use of the mouse and rats in biomedical research has contributed greatly to our understanding of mammalian hematology.^{2,5,12,13,15} This chapter provides

an overview of the applied biology and testing of red blood cells (RBCs), white blood cells (WBCs), platelets, and coagulation pathways in mice and rats, as well as an overview of the bone marrow composition.

Techniques

To ensure that correct values are obtained for hematology, appropriate quality control on sample collection

and handling is required. This is even more important for rodents, for which venipuncture of small vessels is more demanding. The blood volume collected from live rats can be sufficient for hematologic evaluation but in mice, it is typically collected as a terminal procedure. In the mouse, the range of mean circulating blood volume is 6.3–8.0 mL/100 g body weight, with a midpoint blood volume of 7.2 mL/100 g body weight.¹¹ The effect of daily blood collection of even small amounts of blood can be quite marked in the mouse. The blood volume in rats is between 5.0 and 7.1 mL/100 g of body weight.²⁵ In rats, approximately 5.5 mL/kg body weight of blood can be withdrawn safely during a single collection. Collection of up to 40% of blood volume over a period of 24 hours did not lead to increased mortality or morbidity and hematological values returned to control levels within 2 weeks. Younger rats have larger blood volume relative to their body weight than older rats. The choice of site of blood collection varies with experimental design and skill of the phlebotomist (Table 110.1). The sampling site and method must be kept consistent across experiments to avoid creating an unintended experimental variable. As with other species, RBC, WBC, and platelet counts in rodents are generally lower in central veins or in cardiac blood compared with more peripheral sites.²⁷ Sites of interim *in vivo* sampling in rats are the jugular vein, lateral tail vein, tail artery, and sublingual vein, and these procedures can be done with either manual restraint or with anesthesia. Retro-orbital sinus bleeds have also been utilized, but due to animal welfare concerns this technique is not used frequently for *in-life* sampling. Anesthesia is recommended if the retro-orbital sinus technique is used. In mice, limited amounts of blood can be obtained from the lateral tail vein or retro-orbital sinus.^{6,34} A method of sublingual blood sampling in mice has been described.¹⁶ Some facilities are able to obtain a larger volume from the jugular vein with manual restraint or anesthesia. Coating the needle and syringe with a solution of 7.5% EDTA before sampling may help to avoid clotting. Terminal samples are taken at the time of necropsy from the caudal vena cava,

aorta, or via cardiac puncture³¹ and due to the greater volume these samples are easier to use for hematological analysis.

Manual and automated methods can be used with murine or rat blood. Many laboratories conducting hematology tests in rodents use automated hematologic analyzers that have been successfully adapted for analysis of animal samples through the use of animal-specific software. The investigator should always ensure that the accuracy of the hematology instrument has been validated for counting of mouse and rat blood cells.

Preanalytical Considerations

As listed in Table 110.2, many preanalytical factors can affect experimental results and reference intervals in laboratory rodents (see Chapter 13). It is essential that all conditions be kept as similar as possible for control and treated groups of animals. For example, young mice and rats have more reticulocytes and lower RBC counts than slightly older animals. The stress of experimental or husbandry procedures (e.g. blood collection, transportation) may affect WBC counts. The following guidelines are suggested to minimize extraneous influences on hematology parameters and to ensure that experimental results can be interpreted accurately:²⁹ (1) determine that the laboratory's instrumentation is capable of counting rodent RBCs and platelets accurately; (2) minimize stressors such as dosing, transport, and noise prior to blood collection; (3) minimize blood volume collected when repeated collection is required so that previous blood collection will not complicate interpretation at later time-points; (4) collect blood the same way each at each sampling time (same feeding conditions, blood tubes, site of collection, anesthesia, etc); (5) collect blood using the proper blood/anticoagulant ratio; (6) collect and analyze blood either randomly or in replicate order (first animal in each treatment group, then second animal in each treatment group); (7) verify sample quality before analysis and do not use

TABLE 110.1 Blood Collection Sites in Laboratory Rodents^a

Site	Procedure
Heart	T, A
Aorta	T, A
Caudal vena cava	T, A
Orbital sinus	S, A or N
Tail vein	S, A or N
Saphenous vein	S, A or N
Sublingual vein	S, A
Jugular vein (rat only)	S, A or N

^aT, terminal collection procedure; S, survival collection procedure; A, collection procedure under general anesthesia; N, collection procedure not under general anesthesia.

TABLE 110.2 Preanalytical Factors Affecting Hematology Results

Sex
Age
Diet
Housing, supplier
Fasting status
Collection site
Phlebotomist skills
Anticoagulant, sample matrix
Laboratory instrument and methodology
Sample storage
Animal handling prior to collection
Order of collection, processing and analysis

TABLE 110.3a Reference Interval for Hematologic Parameters in Diet-Restricted 7–11 Week Old Sprague Dawley Rats Collected Under Isoflurane Anesthesia⁵

Parameter	Unit	Male range (2.5–97.5%)	Mean (males)	Mean (females)
Red blood cells (RBCs)	$\times 10^6/\mu\text{L}$	7.34–8.85	8.14	8.19
Hemoglobin (Hgb)	g/dL	14.7–17.3	15.9	15.9
Hematocrit (Hct)	%	44.9–51.7	48.5	46.5
Mean cell volume (MCV)	fL	55.1–64.2	59.7	56.9
Mean cell hemoglobin (MCH)	pg	18.6–20.7	19.6	19.5
Mean cell hemoglobin concentration (MCHC)	g/dL	31.3–34.4	32.8	34.3
Red cell distribution width (RDW)	%	11.3–14.2	12.4	11.5
Absolute reticulocytes	$\times 10^6/\mu\text{L}$	0.114–0.399	0.236	0.195
Reticulocytes		1.3–4.94	2.81	2.28
Platelets	$\times 10^3/\mu\text{L}$	903–1594	1159	1146
White blood cells (WBCs)	$\times 10^3/\mu\text{L}$	6.63–20.35	12.43	12.02
Neutrophils	$\times 10^3/\mu\text{L}$	0.37–2.63	0.95	0.72
Lymphocytes	$\times 10^3/\mu\text{L}$	6.10–18.45	10.85	10.79
Monocytes	$\times 10^3/\mu\text{L}$	0.04–0.50	0.20	0.16
Eosinophils	$\times 10^3/\mu\text{L}$	0.02–0.27	0.11	0.15
Basophils	$\times 10^3/\mu\text{L}$	0.01–0.12	0.05	0.05
Large unstained cells	$\times 10^3/\mu\text{L}$	0.04–0.35	0.14	0.12

TABLE 110.3b Reference Interval for Hematologic Parameters in Diet-Restricted 8–16 Week Old Wistar Han Rats Collected Under Isoflurane Anesthesia^a

Parameter	Unit	Male range (2.5–97.5%)	Mean (males)	Female range (2.5–97.5%)	Mean (females)
Red blood cells (RBCs)	$\times 10^6/\mu\text{L}$	7.27–9.65	8.39	7.07–9.03	8.02
Hemoglobin (Hgb)	g/dL	13.7–17.6	15.7	13.7–16.8	15.2
Hematocrit (Hct)	%	39.6–52.5	45	37.9–49.9	43.3
Mean cell volume (MCV)	fL	8.9–57.9	53.5	49.9–58.3	53.8
Mean cell hemoglobin (MCH)	pg	17.1–20.4	18.7	17.8–20.9	19
Mean cell hemoglobin concentration (MCHC)	g/dL	32.9–37.5	34.9	33.2–37.9	35.3
Red cell distribution width (RDW)	%	11.1–15.2	12.7	10.5–14.9	12.2
Absolute reticulocytes	$\times 10^6/\mu\text{L}$	152.3–381.5	238	152.3–381.5	216.6
Platelets	$\times 10^3/\mu\text{L}$	638–1177	904	680–1200	929
White blood cells (WBCs)	$\times 10^3/\mu\text{L}$	1.96–8.25	4.52	1.13–7.49	3.12
Neutrophils	$\times 10^3/\mu\text{L}$	0.22–1.57	0.68	0.15–1.5	0.46
Lymphocytes	$\times 10^3/\mu\text{L}$	1.41–7.11	1.56	0.82–5.66	2.5
Monocytes	$\times 10^3/\mu\text{L}$	0.03–0.18	0.08	0.02–0.16	0.06
Eosinophils	$\times 10^3/\mu\text{L}$	0.01–0.16	0.04	0.01–0.15	0.05
Basophils	$\times 10^3/\mu\text{L}$	0–0.05	0.01	0–0.03	0.01
Large unstained cells	$\times 10^3/\mu\text{L}$	0–0.06	0.02	0–0.04	0.01

^aHistorical Data 2006: Clinical Laboratories, Preclinical Services, Charles River Laboratories, Senneville, Canada.

results for animals with clotted samples, regardless of the size of the clot; (8) keep vials capped until analysis, because small samples evaporate rapidly; and (9) maintain consistent fasting/fed status across groups.¹⁸

Reference Intervals

Reference intervals are available in previous editions of this book^{25,26} and are also provided in this chapter (Tables 110.3a–d). Extensive online reference intervals are also available.⁴⁰ Reference intervals should be used as a tool, but not as the sole guide to determine if values

are normal or abnormal, or to determine whether or not hematologic changes are the result of an experimental procedure.

Erythrocytes

Erythrocyte Morphology

Mature mouse RBCs are round, anucleate, biconcave discs with central pallor, a mean diameter in adults of between 5 and 7 μm , and a cell thickness of 2.1–2.13 μm . Cell volumes vary from 40 to 50 fL. Rat RBCs

TABLE 110.3c Reference Interval for Hematologic Parameters in Diet-Restricted 7–10 Week Old CD-1 Mice Collected Under Isoflurane Anesthesia^a

Parameter	Unit	Male range (2.5–97.5%)	Mean (males)	Female range (2.5–97.5%)	Mean (females)
Red blood cells (RBCs)	×10 ⁶ /μL	7.82–10.11	9.11	7.9–10.12	9.09
Hemoglobin (Hgb)	g/dL	12.6–16.3	14.3	13.2–16.4	14.7
Hematocrit (Hct)	%	42.7–52.9	47.3	43.2–56.3	47.9
Mean cell volume (MCV)	fL	47.6–56.2	52	48.8–58.9	52.7
Mean cell hemoglobin (MCH)	pg	14.7–16.8	15.7	15–16.7	16.1
Mean cell hemoglobin concentration (MCHC)	g/dL	28.7–32.1	30.3	27.9–33.2	30.6
Red cell distribution width (RDW)	%	11.6–13.5	12.7	11.7–14.8	13.2
Absolute reticulocytes	×10 ⁶ /μL	202.9–388.4	269.4	150–477	284.1
Platelets	×10 ³ /μL	1121–1752	1413	630–1559	1188
White blood cells (WBCs)	×10 ³ /μL	0.47–5.16	2.86	0.25–5.18	2.47
Neutrophils	×10 ³ /μL	0.29–1.3	0.64	0.02–1.12	0.41
Lymphocytes	×10 ³ /μL	0.49–3.92	2.12	0.23–4.51	1.97
Monocytes	×10 ³ /μL	0–0.08	0.03	0–0.09	0.02
Eosinophils	×10 ³ /μL	0–0.11	0.06	0–0.21	0.05
Basophils	×10 ³ /μL	0–0.01	0	0–0.01	0
Large unstained cells	×10 ³ /μL	0–0.04	0.01	0–0.06	0.01

^aHistorical Data 2006: Clinical Laboratories, Preclinical Services, Charles River Laboratories, Senneville, Canada.

TABLE 110.3d Reference Interval for Hematologic Parameters in Diet-Restricted 15–36 Week Old C57BL/6 Mice Collected Under Isoflurane Anesthesia^a

Parameter	Unit	Male range (2.5–97.5%)	Mean (males)	Female range (2.5–97.5%)	Mean (females)
Red blood cells (RBCs)	×10 ⁶ /μL	8.98–10.66	10.1	9.23–10.56	9.98
Hemoglobin (Hgb)	g/dL	13.3–15.4	14.6	13.8–15.8	14.6
Hematocrit (Hct)	%	45.8–54.8	50.7	47.6–54.3	50.6
Mean cell volume (MCV)	fL	47.8–54.5	50.2	47.4–55.1	50.8
Mean cell hemoglobin (MCH)	pg	13.8–15	14.4	13.4–15.7	14.7
Mean cell hemoglobin concentration (MCHC)	g/dL	27.3–30.6	28.8	27.5–30.4	29
Red cell distribution width (RDW)	%	12.3–18.5	13.5	12–13.8	12.8
Absolute reticulocytes	×10 ⁶ /μL	177.1–306.1	260.5	204.4–332.3	262.2
Platelets	×10 ³ /μL	321–1866	311	425–1451	1092
White blood cells (WBCs)	×10 ³ /μL	1.22–4.78	2.5	0.28–3.87	1.53
Neutrophils	×10 ³ /μL	0.1–1.66	0.41	0.04–0.61	0.21
Lymphocytes	×10 ³ /μL	0.86–3.79	1.98	0.24–3.42	1.29
Monocytes	×10 ³ /μL	0–0.05	0.01	0–0.02	0.01
Eosinophils	×10 ³ /μL	0–0.07	0.02	0–0.07	0.02
Basophils	×10 ³ /μL	0–0.01	0	0–0.01	0
Large unstained cells	×10 ³ /μL	0–0.09	0.02	0–0.02	0.01

^aHistorical Data 2006: Clinical Laboratories, Preclinical Services, Charles River Laboratories, Senneville, Canada.

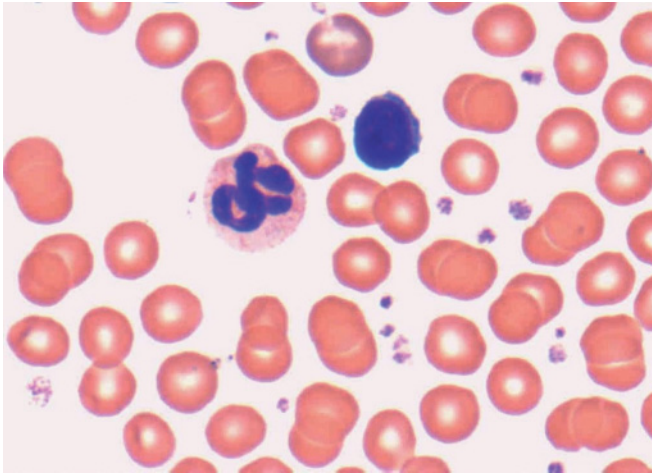


FIGURE 110.1 Blood smear from a healthy rat. Erythrocytes, thrombocytes, neutrophil, small lymphocyte. Modified Wright stain.

(Fig. 110.1) are also anucleated biconcave discs, with an average diameter of $6.2\ \mu\text{m}$ (range $5.7\text{--}7\ \mu\text{m}$). Compared to non-rodent species, mice and rats have more polychromasia and anisocytosis due to higher concentrations of reticulocytes. Moderate anisocytosis of RBCs in rats is common and the diameter of some cells may be up to one-third of the average RBC size. Howell-Jolly bodies are sometimes observed in RBCs and occasionally nucleated RBCs are present in rats and mice.^{13,25}

Erythrocyte Parameters

The RBC count, Hgb concentration, and Hct are generally lower in males than in females and vary with the strain of mouse and rat. Red blood cell counts of mice generally vary from $7 \times 10^6/\mu\text{L}$ to $11 \times 10^6/\mu\text{L}$. Hematocrits in mice generally vary from 35% to 52% and in rat from 37.6% to 51%.¹⁹ Hemoglobin concentrations of mice generally vary from 10 to 17 g/dL and in rat from 11.6 to 16.1 g/dL.¹⁹

Hemoglobin (Hgb) concentrations and hematocrits (Hct) are lower and mean corpuscular volumes (MCV) are higher in rodent neonates. Red blood cells steadily increase from birth (3.7×10^6 RBCs/ μL) to 2-3 months of age in mice (9.34×10^6 RBCs/ μL) and in rats, RBC counts reach adult levels at approximately 4 months of age, with slightly lower counts in males than in females.²⁵ Hemoglobin declines during the early post-natal period (a phenomenon referred to as physiological anemia). Red blood cells of young mice and rats are morphologically variable and have more circulating reticulocytes than older animals. Reticulocyte counts in neonatal mice vary from 40% to 90%. In older rats, relative reticulocyte counts are generally 2-5%, while they can approximate 10-20% in young rats.²⁵ Hematocrits of aged rodents are generally lower than those of young adult animals. The lower Hcts are due to plasma volume expansion, rather than lower RBC mass.

The MCV of mice generally varies from 45 to 55 fL and in rat from 55.1 to 61.5 fL.²⁶ Mean corpuscular hemoglobin (MCH) is not a very useful parameter because it varies proportionally to MCV. Mean corpuscular hemoglobin concentration (MCHC), in conjunction with MCV and RBC distribution width (RDW), is very useful in evaluation of RBC changes in mice and rats. The MCHC in mice generally varies from 30 to 38 g/dL and in rat from 30 to 34 g/dL, depending on species, sex, and age.^{25,26} The RDW indicates variation in RBC size (i.e. anisocytosis) and can only be determined with automated hematology analyzers. Values can be inconsistent among instruments, in part because instruments use different formulas for calculation of RDW.

Erythrocyte Lifespan and Reticulocytes

In mice, the estimated lifespan of RBCs is between 41 and 52 days,¹² while in rats it is between 56 and 69 days.¹⁰ The reticulocyte response to increased demand for RBCs is rapid and robust in mice and rats. Reticulocytes can be counted on blood smears prepared with supravital stains or enumerated on newer automated hematology analyzers by flow cytometric methods (see Chapter 135). Absolute reticulocyte counts (automated methods) in non-anemic mice vary from 150,000 to 300,000/ μL , while percentages of reticulocytes vary from 1% to 5%. In non-anemic adult Wistar rats, absolute reticulocyte counts vary between 154,000 and 271,000/ μL while percentages vary from 1.88% to 3.27%.²¹ Automated reticulocyte counts are a better indicator of erythropoiesis in rats than are reticulocyte percentages.⁸ Some hematology analyzers are capable of subclassifying reticulocytes using size and staining characteristics to determine maturity (low, medium and high reticulocytes). Increases in the immature fraction are thought to have clinical relevance for indicating early erythropoiesis (A. Provencher Bollinger and N. Everds, unpublished observations).

Leukocytes

Evaluation of leukocytes in laboratory rodents includes quantification of total WBC count and of each type of WBC. Leukocyte concentrations in mice and rats demonstrate a diurnal variation. The most frequent WBC in the peripheral blood of the mouse and rat is the lymphocyte (approximately three-fourths of the cells) followed by the neutrophil. Generally, WBC counts of mice and rats vary from 2,000 to 10,000/ μL . White blood cells vary with strain, sex, collection site, anesthesia, time of day, and procedures performed (Table 110.1). Consistent conditions for blood collection are important to minimize these variations. Reporting of WBC differential cell counts as percentages is of limited value³⁷⁻³⁹ because they provide little information about the actual change that is occurring in the leukogram. Therefore, only absolute WBC counts should be interpreted and reported.

Neutrophils

In mice and rats, studies indicate that bone marrow may play an active role in removal of neutrophils from the circulation.³³ In rats, neutrophils preferentially home to bone marrow and macrophages are instrumental in regulating the neutrophil migration. Neutrophils account for between 20% and 30% (mice) and between 12% and 38% (rat) of the total WBC count, and neutrophil counts are higher in male mice than in female mice.

The normal rodent neutrophil has many indentations (many have 5–6 indentations making them look hypersegmented) and criteria used to classify hypersegmented neutrophils in other species should not be used in rats (Fig. 110.1).³ Band forms are infrequently seen in rodents in health, but may be observed during inflammation. Occasional ring forms may be seen and these may increase during accelerated granulopoiesis. Unlike many other species, granulocytes of mice do not express alkaline phosphatase activity.²⁸ Mouse neutrophils have pale cytoplasm with faint pink granules. The nucleus is typically segmented and threads connect the nuclear segments. Nuclear chromatin consists of both pale and condensed DNA (N. Everds, unpublished observation). Rat neutrophils are approximately 11 μm in diameter and have pale cytoplasm with fine, diffuse granules. The nucleus is highly segmented, coiled, or ribbon-like, and has numerous indentations.

Eosinophils

Eosinophils represent 0% to 7% of blood WBCs in mice and 1% to 4% of WBCs in the rat. Mouse eosinophils have a band-shaped and occasionally ring-shaped nucleus that is partially obscured by the presence of ruddy orange to red granules. The granules are large, round, and fairly uniform in size, but have indistinct borders. Rat eosinophils have nuclei that are usually less segmented than neutrophils and contain small, round, reddish granules that fill the cytoplasm (Fig. 110.2). Occasional ring forms are present.

Basophils

Even though rodent basophils are quite distinctive, they are rarely observed on peripheral blood smears. In fact, an incorrect dogma has persisted in the literature and amongst biologists that mice or rats do not have basophils. In contrast to neutrophils or eosinophils, murine basophils lack tertiary granules, but have larger and less numerous mature granules. Basophils should be differentiated from mast cells that can occasionally be seen on blood smears, particularly when cardiac puncture is performed. Mouse and rat basophil nuclei are lobulated and the cytoplasm contains large round purple granules that may be few in number or so numerous that they obscure the nucleus (Fig. 110.3).

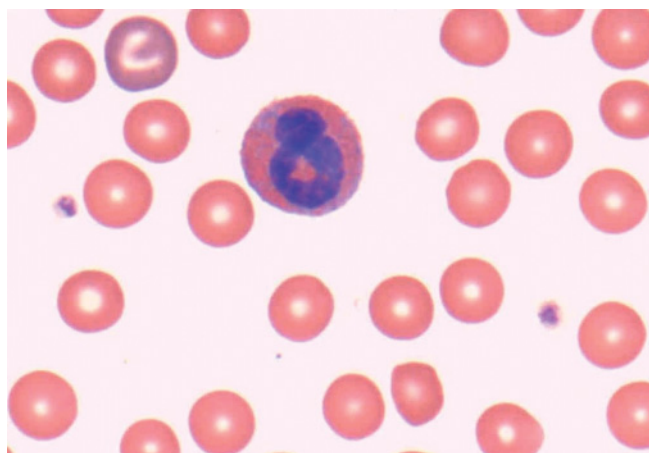


FIGURE 110.2 Blood smear from a healthy rat. Eosinophil. Modified Wright stain.

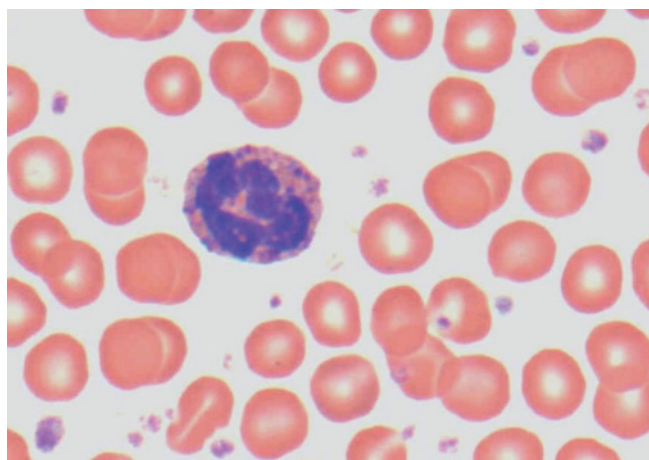


FIGURE 110.3 Blood smear from a healthy rat. Basophil. Modified Wright stain.

Monocytes

Monocytes comprise 0–2% (mice) and 1–6% (rat) of the total WBC count and are the largest leukocytes in both species. In mice, approximately 40% of blood monocytes are in the circulating pool and 60% are in the margined pool. The blood half-life of murine monocytes is estimated to be between 24 hours³⁶ and 40–60 hours.²²

Mouse and rat monocytes are similar to those of other species. Monocytes have pleomorphic nuclei that may be round, indented, or lobular (Fig. 110.4). The cytoplasm is extensive, stains pale gray blue, and often contains vacuoles. Occasionally acidophilic granules are present in the cytoplasm.¹³

Lymphocytes

Lymphocytes comprise 70–80% (mice) and 60–75% (rat) of the differential WBC count. They can represent more than 80% of the total leukocyte count in

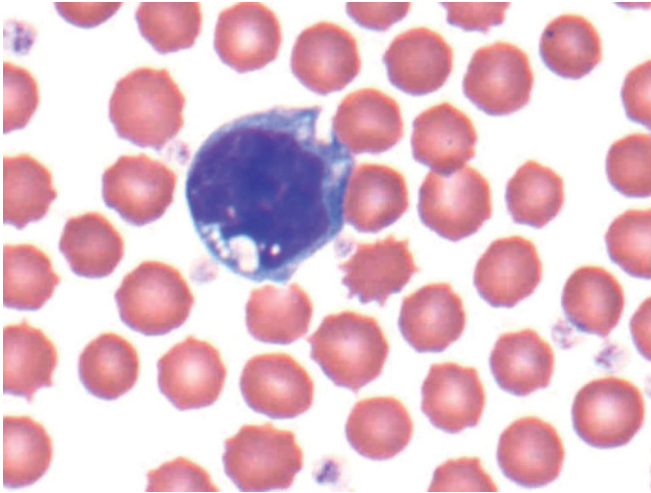


FIGURE 110.4 Blood smear from a healthy mouse. Monocyte. Modified Wright stain.

young mice. Age-dependent variation exists in the neutrophil:lymphocyte ratio, with the proportion of lymphocytes decreasing and proportion of neutrophils increasing as rodents age. In contrast to most other species, lymphocytes make up a significant portion of bone marrow cells in rodents.³

In mice, lymphocytes may be small or large. Small lymphocytes have scant cytoplasm and eccentric nuclei with smudgy chromatin (Figs. 110.1 and 110.5). Large lymphocytes have more extensive cytoplasm. Generally the chromatin is more stippled in large lymphocytes. Occasional clusters of bright pink to magenta granules are observed in lymphocyte cytoplasm. Rats also have small and large lymphocytes. Small lymphocytes have a diameter of about 6 μm with scant cytoplasm that can occasionally contain granules. Large lymphocytes have a diameter up to 15 μm with variable amount of cytoplasm, varying from deep to pale blue, sometimes containing large, dark staining, azurophilic granules.

Alterations in Leukocyte Numbers

Excitement and Stress In mice and rats, excitement increases both lymphocytes and neutrophils. Excitement is associated with release of endogenous catecholamines and results in leukocyte changes that occur within seconds or minutes, due to demargination of neutrophils increasing the circulating WBC count. Stress is a corticosteroid-mediated process, increasing circulating neutrophils, reducing circulating lymphocytes and reducing circulating eosinophils.^{2,32}

Inflammation The inflammatory response in mice and rats, unlike that of other animals, is frequently associated with increased lymphocytes as well as increased neutrophils. In rodents, inflammation may be associated with a neutrophilia in the blood; however, unless

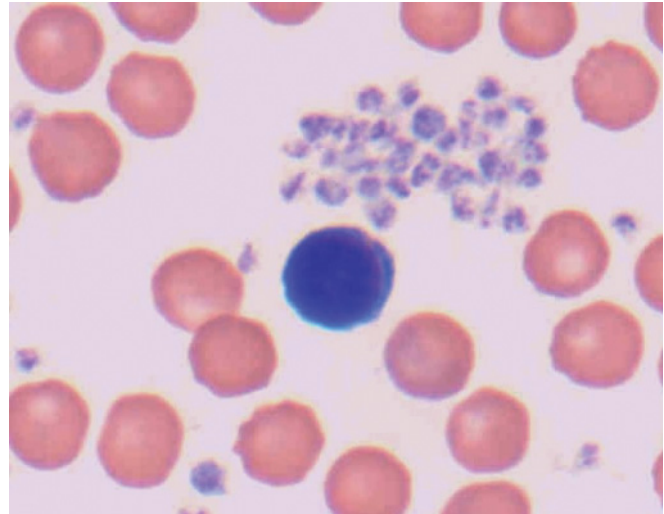


FIGURE 110.5 Blood smear from a healthy mouse. Lymphocyte and platelet aggregate. Modified Wright stain.

the increase in neutrophils is substantial, circulating bands typically are not observed. With chronic inflammation, blood neutrophil counts increase due to increased production. The absolute increase in neutrophils in non-aged rodents is generally less than is seen in dogs or primates. In mice and rats, even small changes in neutrophil counts may be significant. In contrast, marked neutrophilia (up to 50,000/ μL) may be observed in old mice with chronic inflammation.

Neoplasia Infrequently, hematopoietic neoplasms are associated with circulating neoplastic cells (i.e. leukemia). Peripheral blood smears of mice or rats with these neoplasms may have immature or abnormal leukocytes. In the case of lymphocytic leukemia, the neoplastic lymphocytes may be morphologically similar to normal peripheral blood lymphocytes, but the absolute lymphocyte count is variably increased. Hematopoietic and non-hematopoietic neoplasia may also result in decreased leukocyte counts, if the neoplastic population inhibits leukocyte production in bone marrow.

Stem Cell or Stromal Cell Damage Processes or substances that damage hematopoietic stem cells or bone marrow stromal cells may decrease leukocyte counts. Because neutrophils, eosinophils, and monocytes have the shortest half-lives, damage to stem cells or stromal cells is generally first recognized by decreases in these three leukocytes, followed by thrombocytopenia, and then anemia.

Decreased Egress from Blood (Leukocyte Adhesion Defects) Mutations or deficiencies that decrease the ability of leukocytes to egress from the vasculature result in greatly increased circulating WBC counts. Examples include deficiencies in P-selectin, E-selectin, interleukin (IL)-8 receptor, CD18, and/or ICAM-1.⁴

Platelets

Activation and aggregation of platelets are important steps in hemostasis (see Chapter 15). Mouse and rat platelets may be activated spontaneously or in response to stimuli (such as shear stress, adenosine diphosphate, thrombin, and collagen plus epinephrine). The platelet activation state may be strain-dependent. Platelets can be stained with nucleic acid dyes such as thiazole orange and counted using flow cytometry to determine the percentage of young platelets (“reticulated platelets”) in blood (see Chapter 137). Conditions associated with a short platelet lifespan are usually associated with increased reticulated platelets (e.g. activation of platelets in circulation, immune-mediated destruction of platelets).

Mouse platelets have a diameter of 1–4 μm and have a lifespan of approximately 4–5 days (Figs. 110.1 and 110.5). On a blood smear, they are round to oval to elongated.¹² The margins of platelets are indistinct in Wright-Giemsa-stained smears. The cytoplasm stains faintly pink to gray and intensely blue angular granules are usually observed near the center of the cell. Red granules may also be present. Rat and mouse platelets have similar morphology. Round platelets as large as RBCs and platelet clumps are quite frequently observed.

Platelet counts in rodents are high compared with larger domestic species. Platelet counts in mice average $900 \times 10^3/\mu\text{L}$ to $1600 \times 10^3/\mu\text{L}$ ¹² and in rats, average between $430 \times 10^3/\mu\text{L}$ and $1450 \times 10^3/\mu\text{L}$.²⁵ Due to the small size of rodent platelets, some hematology analyzers are only capable of measuring larger rodent platelets and thus underestimate the platelet count. In addition, rodent platelets frequently become activated and clump *in vitro*, making platelet counts inaccurate.²⁰ Mean platelet volume varies between 4 and 6 fL; however, the variability in platelet size is greater than that of RBCs. Platelet size can also be subjectively assessed on the peripheral blood smear. The platelet crit (PCT), a measure of total platelet mass, is a parameter provided on some hematology analyzers. This parameter is analogous to the hematocrit, and is determined by multiplying the platelet count by the mean platelet volume. It has been proposed that PCT is more indicative of hemostatic capability than is the platelet count.²⁴ Platelet distribution width (PDW) is analogous to the RDW. Like RDW, PDW increases with variability in platelet size and is a measure of platelet anisocytosis.

Changes in platelet parameters

Increased platelet counts are generally due to increased platelet production. Accelerated platelet production is frequently associated with the presence of giant platelets in the blood. Platelet counts show modest increases during accelerated erythropoiesis, during some inflammatory diseases, and in iron deficiency. Decreased platelet numbers are a common hematologic finding in rodents. The most frequent cause is artifactual decreases secondary to blood collection difficulties. Mouse plate-

lets readily aggregate and instrument-generated platelet counts will underestimate true platelet counts in the presence of platelet clumping. True thrombocytopenia is caused by decreased production and shortened half-life, usually due to activation and consumption.

Coagulation

Murine hemostatic factors have been reviewed.^{4,17} The plasma protein factors involved in coagulation in mice and rats are similar to other species with some differences: prothrombin and factor IX activities of CAF1 mice are about half those of humans (see Chapter 15).⁹

Coagulation tests are commonly performed in rats, but less commonly conducted on mice, due to difficulty in sample collection (see Chapter 15). A few studies have determined reference values for coagulation times in mice. General reference intervals for bleeding time, prothrombin time, activated partial thromboplastin time, and fibrinogen are provided in Table 110.4. The anatomic site used for blood collection may influence results of coagulation tests. For example, in rats, prothrombin time and partial thromboplastin time are significantly increased and more variable in blood collected from the orbital venus plexus than in samples from the posterior vena cava. Other coagulation tests such as D-dimer, antithrombin, thrombin time, and specific factor assays may be useful when studying models of coagulopathy; however, published literature is limited.

Altered coagulation times in rodents and particularly in mice are most frequently caused by problems with sample collection, such as improper ratio of anticoagulant to blood, technical inexperience, or difficulty in collecting blood from animals with compromised health (see Chapter 15). When interpreting prolonged coagulation times, it is important to recognize that these tests are insensitive for detecting small changes

TABLE 110.4 General Hemostasis Parameters in Mice and Rats

Test	Mouse	Rat
In vivo		
Bleeding time (minutes)	<1 ^a	2–5 ^b
In vitro		
Prothrombin time (seconds)	7–19 ^a	4–8 ^{c,d} 15–19 ^e
Activated partial thromboplastin time (seconds)	16–110 ^{a,f}	17–25 ^{c,d} 12–20 ^e
Fibrinogen (mg/dL)	114–324 ^a	150–230 ^{c,d}

^aRef. 12.

^bRef. 4.

^cNorway rat, males and females.

^dRef. 40.

^eWistar rat, males and females.

^fWide intervals suggest APTT is highly sensitive to laboratory method and/or mice strains.

in coagulation factors. Therefore, small but consistent prolongations of coagulation times may indicate significant changes in coagulation factor concentrations and should be investigated for possible coagulation factor effects. Changes in coagulation times become significant when multiple animals in a treatment group are involved.

Bone Marrow Evaluation and Cytology

The overall examination of bone marrow smears in rats does not differ from bone marrow examination of other animal species. Evaluation should be made in light of available clinical information, physical signs, hematological data, and results of histological examination of hematopoietic organs. The accurate interpretation of bone marrow smears necessitates a systematic, stepwise approach. Indication for bone marrow collection in rodents, slide preparation and evaluation are described in Chapter 132.

Cytological evaluation of bone marrow has been described in rat and is similar in mice.^{1,3,14,35} The ratio of myeloid to erythroid cells (M:E) in mouse bone marrow varies from 0.8:1 to 2.79:1 and averages 1.5:1.^{14,23} These ratios are similar when determined by flow cytometry or by microscopy,¹⁴ and are dependent on strain and age. Lymphocytes comprise one-fifth to one-third of bone marrow cells in mice and rats.^{3,14,23} Megakaryocytes are about twice as numerous in murine bone marrow as in humans. In rodents, mature megakaryocytes are multinucleated and nuclei are frequently fused into a lobulated mass. A peculiarity in rats is the presence of megakaryocytes with mild pleomorphism, characterized by separated (i.e. non-fused) nuclei, with variable nuclear-to-cytoplasmic ratios.³ Emperipolesis (passage of leukocytes through the cytoplasm of megakaryocytes) is occasionally observed in bone marrow from normal mice and rats. Megakaryocytes are not usually counted but assessed as present in sufficient or insufficient number, with adequate or inadequate maturation and normal or abnormal morphology. Maturation of the megakaryocytic series should be evaluated by assessing the number of mature versus immature forms and should be orderly, with lower numbers of immature forms than mature.

The erythroid cell line in rodents resembles those of other species (Fig. 110.6). The erythroid maturation sequence consists of rubriblasts, maturing into prorubricytes, then rubricytes that mature into basophilic rubricytes, polychromatophilic rubricytes and metarubricytes. After the metarubricyte stage, the nucleus is expelled and the cell matures into polychromatophilic RBCs (reticulocytes) then to mature RBCs. Reported percentages for erythroid precursors vary from 20% to almost 40% of all nucleated cells.³ A low number of binucleated metarubricytes or metarubricytes with irregular nuclear shape may be observed in healthy rats.

Granulocyte precursors in rodents resemble those of other species but also have unique features, making

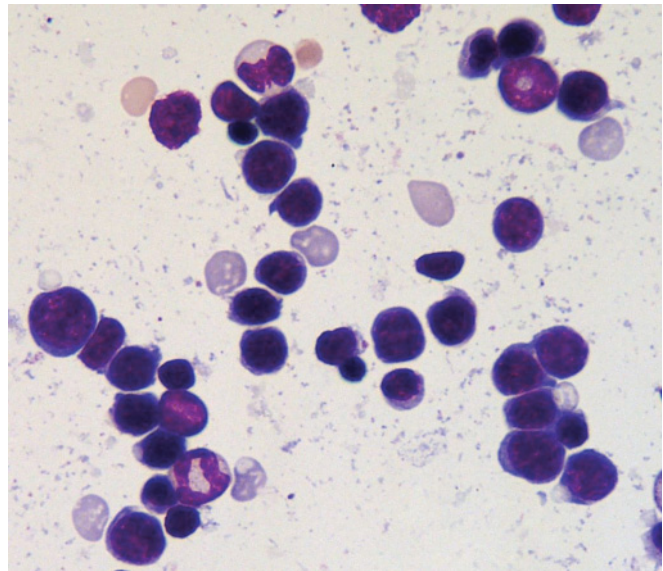


FIGURE 110.6 Erythroid precursors. (Bone marrow smear from healthy rat; May-Grünwald-Giemsa stain; 100× objective.)

identification sometimes challenging. Reported percentages of myeloid precursors vary from 33.6% to 52%.³ The earliest stage that can be recognized by light microscopy is myeloblasts, progressing to promyelocytes, then myelocytes, metamyelocytes, band granulocytes, and mature cells. In rodents and non-rodent species, the nucleus of the myelocyte is usually round to slightly oval and, as maturation progresses, the indentation of the nucleus goes from round to horse-shoe-shaped, to segmented. In mice and rats, precursors can take a "ring form" from the promyelocyte stage.³ With cell maturation, the hole becomes larger and the nucleus thinner until a very thin rim of nuclear material is present in the band or non-segmented stage (Figs. 110.7 and 110.8). Ring forms can be seen in neutrophils and eosinophils, but not in basophil lines.

Monocyte and macrophage precursors are present in low number in the bone marrow, unless there is a disease process making this cell line overrepresented. Monocyte precursors are difficult to differentiate from early granulocytic cells.

The percentage of lymphocytes in bone marrow of healthy mice and rats is quite variable, being reported to be between 7.34% and 21.1% of the total nucleated cell count.³ Lymphocyte counts may vary with age, younger animals having more lymphocytes than adults.³⁰ Depending on the stain used, it can be challenging to clearly differentiate small mature lymphocytes from metarubricytes. Lymphoid precursors are present in low number and it can be difficult to differentiate precursors from other early myeloid precursors on Romanowsky-stained preparations. The use of flow cytometry in differentiating bone marrow cells can give a more accurate count of lymphoid cells than microscopic differential cell counting.^{7,40} Plasma cells are present in low numbers, unless there is a proliferation due to a pathologic process.

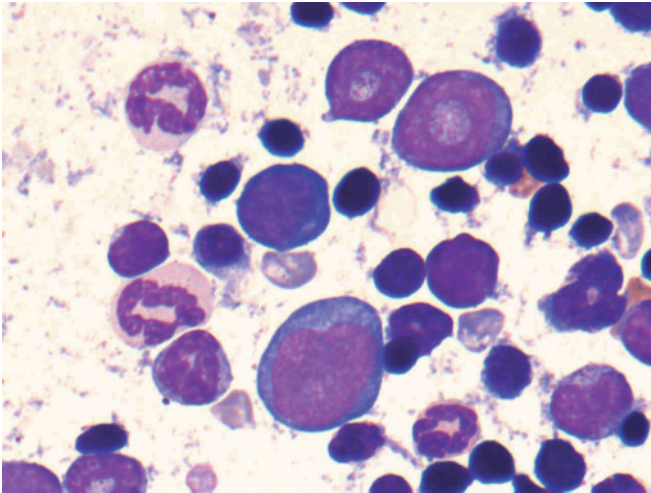


FIGURE 110.7 Granulocytic precursors; ring forms. Few small dark erythroid forms noted. (Bone marrow smear from healthy mouse; May-Grünwald-Giemsa stain; 100× objective.)

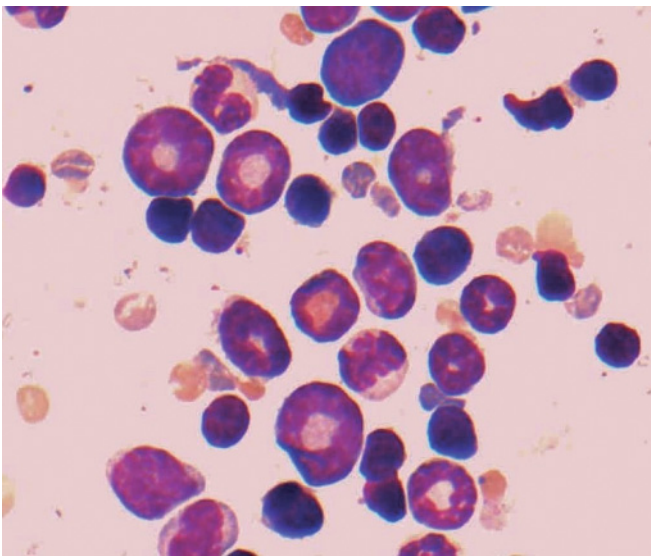


FIGURE 110.8 Granulocytic precursors; ring forms. Several small dark erythroid forms noted. (Bone marrow smear from healthy rat; May-Grünwald-Giemsa stain; 100× objective.)

Many other cells can be observed in bone marrow of rodents as in other species. These include mast cells, osteoblasts, osteoclasts, and endothelial cells. Mast cells can be present in very high number in rats, making cytological evaluation of smears difficult. Mast cells can degranulate and create a thick granular background. Mast cells are usually not counted in differential cell counts or M:E ratios. Osteoblasts and osteoclasts can be observed in low numbers and are not counted. These cells should not be confused with megakaryocytes. Endothelial cells can be seen when small capillaries are smeared on the preparation.

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HEMATOLOGY OF LABORATORY RABBITS (*ORYCTOLAGUS CUNICULUS*)

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Blood Collection

Blood is usually collected from the marginal ear vein of rabbits. The rabbit can be restrained in a commercial rabbit restrainer, a zippered cat bag (available at most veterinary clinics), or by securely wrapping the rabbit in a large towel.²⁴ The fur covering the marginal ear vein is plucked, which aids in visualization and dilation of the vessel. By warming the ear with a warm washcloth, warming it next to an incandescent light bulb, and/or stroking the central artery from the base to the tip of the ear, dilation of the vein will be enhanced. Pretreatment with the tranquilizer acetylpromazine (0.25 mL subcutaneously), will also result in dilation of peripheral veins including the marginal ear vein. A 23- to 25-gauge needle can be used when collecting blood. The vacuum from a Vacutainer tube frequently collapses the vessel, and attempting rapid withdrawal with a syringe may also collapse the vessel and prevent the free flow of blood. Alternatively, blood can be collected as it flows from the hub of a needle inserted in the marginal ear vein. A peristaltic pump or an Erlenmeyer flask to which a vacuum is applied using a vacuum pump, may facilitate blood collection from the marginal ear vein.³⁴ When using the flask, the fur over the vessel is plucked, the vessel is pricked with a sterile lancet, and the ear is inserted into the flask. A rubber stopper on the outlet port of the flask is inserted into an appropriately sized centrifugation tube for collection of nonsterile blood. Although the central artery of the ear can be used for blood collection by this technique, it should be used with caution, because formation of a hematoma is a potential sequela.

Other blood collection sites include the central artery of the ear, jugular vein, the lateral saphenous vein, and direct cardiac puncture (in an anesthetized animal).^{6,24} An 18-gauge or larger hypodermic needle, between 1.5 and 2 inches (3.8–5.1 cm) in length, attached to tubing from a human blood collection set, can be inserted in the third left intercostal space, about 4 mm lateral to the sternum, until it penetrates the heart and blood is seen flowing into the tube. Cardiac blood collection is usually

reserved for terminal exsanguination procedures, and should not be used for clinical patients because it can result in myocardial damage, hemothorax, pericardial tamponade, or death. The animal should be adequately anesthetized before cardiac blood collection is attempted. Generally, collection of blood from the jugular vein also requires sedation or light anesthesia (i.e. gas anesthesia administered with a nose cone).

The total blood volume in rabbits was estimated as 4.5–8.1% of total body weight, or approximately 53.8 ± 5.2 mL/kg.^{24,27–29} However, Jain cautioned against introducing inaccuracies by expressing blood volume on the basis of actual body weight, and instead suggested that blood volume be correlated with lean body weight or body surface area.¹³ The recommended maximum volume of blood that can be safely collected during one bleeding is 7.7 mL/kg of body weight.²⁹

Morphology and Numbers of Blood Cells

Reported hematologic values for New Zealand white (NZW) rabbits (*Oryctolagus cuniculus*) are provided in Tables 110.5 and 110.6.^{2,16,24,30} Hematologic values reported for some other breeds or species of rabbits are provided in Tables 110.7 and 110.8.^{4,7,8,11,16,18,21,40} An excellent discussion of the sources of variation found in hematologic values was provided by McLaughlin and Fish and others.^{24,25}

Erythrocytes

The rabbit RBC is a biconcave disc with an average diameter between 6.7 and 6.9 μ m and an average thickness of 2.15–2.4 μ m.^{8,13,24,30} There is marked anisocytosis of the RBCs in rabbits, with microcytes of one-quarter the diameter of normal-sized RBCs sometimes seen. Schermer described the appearance of numerous thorn apple-shaped forms in blood smears as characteristic of rabbit blood, and observed that polychromasia was found in 1–2% of RBCs (Fig. 110.9).³³ Reticulocytes accounted for 1–7% of RBCs in adult animals and were determined to be $2 \pm 0.5\%$ in males and $3 \pm 0.5\%$ in females. One- to two-month-old New Zealand White rabbits were found to have reticulocyte counts of $7.4 \pm 4.7\%$, with a 50% reduction in those counts occurring during the third month of life and a subsequent smaller decrease to $3.0 \pm 1.3\%$ in adult rabbits.¹¹ Reticulocyte counts were found to increase after

TABLE 110.5 Referenced Erythrocyte Parameters of the New Zealand White (NZW) Rabbit (*Oryctolagus cuniculus*)

Reference	Gender ^b	RBC ($\times 10^6/\mu\text{L}$)		PCV (%)		Hgb (g/dL)		MCV (fL)		MCH (pg)		MCHC (%)	
		Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range
33	NS	5.25	4–6.4	—	—	12.4	8.4–15.5	—	—	—	—	—	—
16	NS	6.2	—	39	—	13.4	—	60	—	23	—	35	—
28	M/F (adult)	—	5.11–7.94	—	37–50	—	9.8–17.4	—	57.8–65.4	—	17.1–23.5	—	28.7–37
23	NS (1 yr)	7.73 \pm 0.78	—	49.08 \pm 3.98	—	15.97 \pm 1.3	—	63.62 \pm 2.47	—	20.7 \pm 1.07	—	32.52 \pm 1.04	—
	NS (adult)	7.79 \pm 0.51	—	47.58 \pm 2.89	—	15.95 \pm 1.18	—	61.08 \pm 2.45	—	20.48 \pm 1.1	—	33.54 \pm 1.2	—
29	M	6.7 \pm 0.62	5.46–7.94	41.5 \pm 4.25	33–50	13.9 \pm 1.75	10.4–17.4	62.5 \pm 2.0	58.5–66.5	20.7 \pm 1.0	18.7–22.7	33.5 \pm 1.85	33–50
	F	6.31 \pm 0.6	5.11–6.51	32.2 \pm 4.4	31.0–48.6	12.8 \pm 1.5	9.8–15.8	63.1 \pm 1.92	57.8–65.4	20.3 \pm 1.6	17.1–23.5	32.2 \pm 1.74	28.7–35.7
32	M (3 mo)	5.3 \pm 0.4	—	34 \pm 2	—	11.2 \pm 0.7	—	65 \pm 3	—	21 \pm 1	—	33 \pm 1	—
	F (3 mo)	5.4 \pm 0.6	—	36 \pm 3	—	11.7 \pm 1	—	67 \pm 4	—	22 \pm 1	—	33 \pm 1	—
37	NS	—	4–7	—	30–50	—	8–15	—	—	—	—	—	—
12	M (1–2 mo)	5.64 \pm 0.49	—	40.5 \pm 2.4	—	11.8 \pm 0.8	—	72.2 \pm 5.1	—	21 \pm 1.3	—	28.4 \pm 4.2	—
	F (1–2 mo)	5.5 \pm 0.62	—	40.3 \pm 2.2	—	11.5 \pm 0.8	—	73.9 \pm 6.4	—	21.1 \pm 1.6	—	28.5 \pm 0.9	—
	M (3 mo)	6.24 \pm 0.24	—	42.5 \pm 1.6	—	13.4 \pm 0.5	—	68.1 \pm 1.9	—	21.5 \pm 0.6	—	31.4 \pm 0.9	—
	F (3 mo)	6.02 \pm 0.23	—	41.4 \pm 2.5	—	12.6 \pm 0.7	—	68.7 \pm 2.5	—	20.9 \pm 0.8	—	30.4 \pm 1	—
	M (4–6 mo)	6.34 \pm 0.39	—	43.3 \pm 2.6	—	13.9 \pm 1.1	—	68.2 \pm 4.1	—	21.9 \pm 1.5	—	32 \pm 1.2	—
	F (4–6 mo)	6.32 \pm 0.43	—	43.0 \pm 2.3	—	13.5 \pm 0.9	—	68.2 \pm 3.0	—	21.4 \pm 1.2	—	31.4 \pm 1.1	—
	M (7–12 mo)	6.03 \pm 0.3	—	42.4 \pm 1.6	—	13.7 \pm 0.6	—	70.9 \pm 2.3	—	22.7 \pm 0.8	—	32 \pm 8	—
	F (7–12 mo)	5.95 \pm 0.43	—	41.7 \pm 3.2	—	13.1 \pm 1.0	—	70.2 \pm 3.1	—	22.1 \pm 1.1	—	31.4 \pm 1.2	—
	M (1–2 yrs)	6.34 \pm 0.7	—	42.7 \pm 1.8	—	13.2 \pm 1.0	—	67.9 \pm 5.6	—	21 \pm 1.1	—	31 \pm 1.8	—
	F (1–2 yrs)	5.96 \pm 0.54	—	40.8 \pm 3.5	—	12.7 \pm 1.3	—	68.5 \pm 2.7	—	21.4 \pm 1	—	31.3 \pm 1.4	—
40	M ($n = 98$)	6.75 \pm 0.533	—	40.4 \pm 3.05	—	13.7 \pm 1.0	—	59.9 \pm 2.78	—	20.4 \pm 0.97	—	34.0 \pm 0.52	—
	F ($n = 98$)	6.22 \pm 0.484	—	37.8 \pm 2.31	—	12.8 \pm 0.78	—	60.9 \pm 2.4	—	20.8 \pm 0.93	—	34.1 \pm 0.61	—
2	F (2.9–4.4 kg)	5.7 \pm 0.4	—	36 \pm 3	—	12.1 \pm 1.0	—	62 \pm 1	—	21	—	34	—
9	NS (4–7 mo)	6.0 \pm 0.6	3.7–7.5	38 \pm 3.1	26.7–47.2	12.8 \pm 1.0	8.9–15.5	63.7 \pm 3.1	58–79.6	21.4 \pm 1.3	19.2–29.5	33.6 \pm 0.6	31.1–37
16	M	6.4 \pm 0.4	—	43 \pm 2	—	14 \pm 0.6	—	65 \pm 4	—	21 \pm 1	—	32.5 \pm 0.4	—
	F	6.0 \pm 0.6	—	39 \pm 2	—	12.7 \pm 0.6	—	66 \pm 2	—	22 \pm 1	—	32.8 \pm 0.3	—
7	NS	—	4–7.2	—	36–48	—	10.0–15.5	—	—	—	—	—	—
14	NS	—	4–7	—	36–48	—	10.0–15.5	—	—	—	—	—	—
10	NS	—	5.1–7.9	—	33–50	—	10.0–17.4	—	57.8–66.5	—	17.1–23.5	—	29–37
Covance	M	6.0	0.3 SD	38.3	2.0 SD	12.6	0.6 SD	64	2 SD	21	1 SD	33	1 SD
2008 ^a	F	5.8	0.4 SD	38.2	2.1 SD	12.2	0.6 SD	66	3 SD	21	1 SD	32	1 SD

^aData supplied by Covance laboratories, www.covance.com.^bNS, not specified.

SD, standard deviation.

TABLE 110.6 Referenced Leucocyte parameters of the New Zealand White (NZW) Rabbit (*Oryctolagus cuniculus*)

Reference	Gender ^b	WBC ($\times 10^9/\mu\text{L}$)		Heterophils (%)		Lymphocytes (%)		Eosinophils (%)		Basophils (%)		Monocytes (%)	
		Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range
33	NS	8	5.2–12	—	8–50	—	20–90	—	1–3	—	0.5–30	—	1–4
16	NS	8.1	—	32	—	63	—	1.3	—	2.4	—	4.1	—
28	NS	—	5.2–12.5	—	36.4–54	—	28–52.1	—	0.5–3.5	—	2.4–7.5	—	4.0–13.4
23	NS (1yr)	4.91 \pm 2.19	—	—	—	54.2 \pm 11.6	—	4.5 \pm 3.6	—	—	—	6.3 \pm 3.5	—
	NS (adult)	7.46 \pm 3.15	—	—	—	42.7 \pm 19.8	—	2.2 \pm 2.0	—	—	—	4.3 \pm 3.0	—
29	M	9.0 \pm 1.75	5.5–12.5	46 \pm 4	38–54	39 \pm 5.5	28–50	2 \pm 0.75	0.5–3.5	5 \pm 1.25	2.5–7.5	8 \pm 2	4–12
	F	7.9 \pm 1.35	5.2–10.6	43.4 \pm 3.5	36.4–50.4	41.8 \pm 5.15	31.5–52.1	2 \pm 0.6	0.8–3.2	4.3 \pm 0.95	2.4–6.2	9 \pm 2.2	6.6–13.4
32	M (3 mo)	9.7 \pm 3.3	—	30	—	60	—	1	—	3.1	—	3.1	—
	F (3 mo)	7.7 \pm 2.2	—	25	—	64	—	0	—	3.9	—	5.5	—
37	NS	—	6–12	—	20–60	—	20–50	—	0–5	—	0–1	—	1–10
12	M (1–2 mo)	5.99 \pm 1.98	—	34.7 \pm 11.7	—	57.7 \pm 13.1	—	0.8 \pm 0.6	—	2.0 \pm 1.7	—	5.5 \pm 7.0	—
	F (1–2 mo)	5.38 \pm 1.85	—	31.9 \pm 12.2	—	61.1 \pm 12.5	—	0.9 \pm 0.9	—	2.3 \pm 2.2	—	3.7 \pm 2.6	—
	M (3 mo)	8.45 \pm 1.34	—	28.9 \pm 8.4	—	65.7 \pm 9.2	—	1.6 \pm 0.8	—	0.8 \pm 0.6	—	5.6 \pm 7.5	—
	F(3 mo)	9.1 \pm 3.54	—	28.8 \pm 10	—	67.5 \pm 10.3	—	0.9 \pm 0.8	—	1.5 \pm 0.8	—	1.4 \pm 0.9	—
	M (4–6 mo)	7.71 \pm 1.08	—	27.6 \pm 10.4	—	68.5 \pm 11.1	—	0.8 \pm 0.8	—	1.4 \pm 1.3	—	1.7 \pm 1.4	—
	F (4–6 mo)	7.69 \pm 1.6	—	28.9 \pm 10.4	—	63.9 \pm 10.4	—	1.5 \pm 0.9	—	2.5 \pm 1.8	—	2.7 \pm 2.3	—
	M (7–12 mo)	8.99 \pm 1.75	—	27.9 \pm 8	—	62 \pm 16.9	—	0.8 \pm 0.7	—	3.0 \pm 2.6	—	2.5 \pm 2.4	—
	F (7–12 mo)	7.69 \pm 1.8	—	30 \pm 9.7	—	62.8 \pm 11.8	—	1.2 \pm 1.1	—	2.4 \pm 2.0	—	3.6 \pm 2.6	—
	M (1–2yrs)	10.0 \pm 2.85	—	47 \pm 5.9	—	44.5 \pm 7	—	1.5 \pm 1	—	2.3 \pm 2.3	—	4.9 \pm 4.5	—
	F (1–2yrs)	9.72 \pm 3.3	—	44.7 \pm 14.6	—	45.6 \pm 14.4	—	2.0 \pm 1.6	—	3.3 \pm 2.2	—	4.8 \pm 2.5	—
40	M (2–7 mo)	9.5 \pm 2.07	—	32 \pm 10.95	—	62 \pm 13.2	—	1 \pm 0.8	—	2 \pm 1.7	—	1 \pm 1.4	—
	F (2–7 mo)	8.4 \pm 2.24	—	34 \pm 10.7	—	61 \pm 11.3	—	1 \pm 1.3	—	3 \pm 1.8	—	1 \pm 1.1	—
2	F (2.9–4.4 kg)	8.1 \pm 2.7	—	32 \pm 15	—	68 \pm 15	—	—	—	—	—	—	—
9	NS	9.2 \pm 2.2	5.2–16.5	—	—	—	—	—	—	—	—	—	—
16	M (1–2yrs)	6.8 \pm 1.2	—	26 \pm 7	—	58 \pm 8	—	0.5 \pm 0.4	—	3.2 \pm 0.8	—	6.0 \pm 2.4	—
	F (1–2yrs)	5.6 \pm 0.9	—	35 \pm 3	—	47 \pm 7	—	1.2 \pm 0.8	—	5.0 \pm 2.5	—	6.6 \pm 2.4	—
7	NS	—	7.5–13.5	—	20–35	—	55–80	—	0–4	—	2–10	—	1–4
14	NS	—	9–11	—	20–75	—	30–85	—	0–4	—	2–7	—	1–4
10	NS	—	5.2–12.5	—	20–75	—	30–85	—	1–4	—	1–7	—	1–4
Covance (2008) ^a	M	8.1	2.2 SD	37	11 SD	58	11 SD	0	0 SD	2	1 SD	3	2 SD
	F	8.2	2.6 SD	43	12 SD	53	13 SD	2	NS	2	2 SD	3	2 SD

^aData supplied by Covance laboratories, www.covance.com.

^bNS, not specified.

SD, standard deviation.

TABLE 110.7 Referenced Erythrocyte Parameters for Some Other Rabbit Breeds/Species

Reference	Breed/Species	Gender ^b	RBC ($\times 10^6/\mu\text{L}$)		PCV (%)		Hgb (g/dl)		MCV (fL)		MCH (pg)		MCHC (%)	
			Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range
18	Dutch Belted (<i>Lepus europaeus</i>)	M	5.45 \pm 0.13	—	41.18 \pm 0.71	—	13.98 \pm 0.25	—	—	—	—	—	—	—
		F	5.3 \pm 0.14	—	41.79 \pm 0.91	—	14.21 \pm 0.35	—	—	—	—	—	—	—
29	Dutch Belted (<i>Lepus europaeus</i>)	NS	—	4.8–6.3	—	34.8–48.9	—	12.2–16.3	—	62.7–88.1	—	22.0–29.4	—	28.5–38.1
Covance (2008) ^a	Dutch Belted (<i>Lepus europaeus</i>)	M	6.4	0.5 SD	36.2	12.4 SD	13.4	1.1 SD	62	1 SD	21	1 SD	34	1 SD
		F	5.5	0.7 SD	36.4	3.4 SD	11.6	0.9 SD	63	3 SD	22	4 SD	34	2 SD
29	Polish White (<i>Lepus europaeus</i>)	NS	—	4.6–6.32	—	36.7–43.5	—	11.9–16.3	—	67.7–80.3	—	22.0–30.1	—	29.7–40.6
4	Jackrabbit (<i>Lepus californicus</i>)	NS	—	6.59–8.56	—	42–53	—	13.7–17.5	—	57.6–70	—	18.1–23.1	—	28.8–36.8
		NS (1yr)	—	6.17–9.29	—	41.2–57	—	13.4–18.6	—	58.7–68.6	—	18.6–22.8	—	30.4–34.6
29	Jackrabbit (<i>Lepus californicus</i>)	NS (adult)	—	6.77–8.81	—	41.8–53.4	—	13.6–18.3	—	56.2–66	—	18.3–22.7	—	31.1–35.9
		NS (<1yr)	7.73 \pm 0.78	—	49.08 \pm 3.98	—	15.97 \pm 1.3	—	63.62 \pm 2.47	—	20.7 \pm 1.07	—	32.52 \pm 1.04	—
12	Jackrabbit (<i>Lepus californicus</i>)	NS (adult)	7.79 \pm 0.51	—	47.58 \pm 2.89	—	15.98 \pm 1.18	—	61.08 \pm 2.45	—	20.48 \pm 1.1	—	33.54 \pm 1.2	—
		NS (autumn)	—	—	34	—	—	—	—	—	—	—	—	—
11	Eastern Cottontail (<i>Sylvilagus floridanus</i>)	NS (winter)	—	—	45	44–54	—	—	—	—	—	—	—	—
		NS (spring)	—	—	42	31–46	—	—	—	—	—	—	—	—
		NS (summer)	—	—	40	31–47	—	—	—	—	—	—	—	—
20	Eastern Cottontail (<i>Sylvilagus floridanus</i>)	NS	—	—	37.2 \pm 6.7	18–49	—	—	—	—	—	—	—	—

^aData supplied by Covance laboratories, www.covance.com

^bNS, not specified.

SD, standard deviation.

TABLE 110.8 Referenced Leukocyte Parameters for Some Other Rabbit Breeds/Species

Reference	Breed/Species	Gender ^b	WBC ($\times 10^3/\mu\text{L}$)		Heterophils (%)		Lymphocytes (%)		Eosinophils (%)		Basophils (%)		Monocytes (%)	
			Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range
18	Dutch Belted (<i>Lepus europaeus</i>)	M	7.14 \pm 0.74	—	36.29 \pm 2.94	—	58.47 \pm 2.79	—	2.0 \pm 0.57	—	1.35 \pm 0.43	—	2.47 \pm 0.4	—
		F	7.01 \pm 0.48	—	23.42 \pm 2.51	—	71.53 \pm 3.01	—	0.89 \pm 0.2	—	1.74 \pm 0.58	—	2.37 \pm 0.43	—
29	Dutch Belted (<i>Lepus europaeus</i>)	NS	—	4–13	—	30–50	—	28.5–52.5	—	0.5–5.0	—	2–8	—	2–16
		M	9	3.5 SD	31	10 SD	65	10 SD	—	—	2	1 SD	2	1 SD
Covance (2008) ^a	Dutch Belted (<i>Lepus europaeus</i>)	F	6.1	2.8 SD	39	9 SD	56	8 SD	2	NS	1	1 SD	3	2 SD
29	Polish White (<i>Lepus europaeus</i>)	NS	—	7.45–13.3	—	16.4–49.9	—	73–90.0	—	0.13–1.63	—	1.13–3.63	—	0.73–3.25
4	Jackrabbit (<i>Lepus californicus</i>)	NS	—	2.2–14.7	—	13.0–81.5	—	25–83	—	0–8	—	0–1.5	—	2–10
29	Jackrabbit (<i>Lepus californicus</i>)	NS (1 yr)	—	2.7–7.1	—	11.8–57.4	—	31.0–77.4	—	0.9–8.1	—	0–1	—	2.8–9.8
		NS (adult)	—	3.31–10.8	—	11.4–71.8	—	22.9–81.3	—	0.2–4.2	—	0–0.9	—	1.3–7.3
12	Jackrabbit (<i>Lepus californicus</i>)	NS (<1 yr)	4.91 \pm 2.2	—	34.6 \pm 11.4	—	54.2 \pm 11.6	—	4.5 \pm 3.6	—	0.4 \pm 0.6	—	6.3 \pm 3.5	—
		NS (adult)	7.46 \pm 3.15	—	50.4 \pm 21.4	—	42.7 \pm 19.8	—	2.2 \pm 2.0	—	0.4 \pm 0.5	—	4.3 \pm 3.0	—
11	Eastern Cottontail (<i>Sylvilagus floridanus</i>)	NS (autumn)	—	—	51	—	43.5	—	1	0–9.5	1.5	—	1.5	—
		NS (winter)	—	—	28.5	21–45.5	62.5	48–71.5	1	1–1.5	1.5	0.5–2.5	2	1–2.5
		NS (spring)	—	—	48	47–69.5	43	25.5–45.5	0.5	0–1	2	1.0–2.5	2	1.5–3.5
		NS (summer)	—	—	43	5–67	53	29–94	0	0–1	0.75	0–3	2.7	1.0–5.5

^aData supplied by Covance laboratories, www.covance.com

^bNS, not specified.

SD, standard deviation.

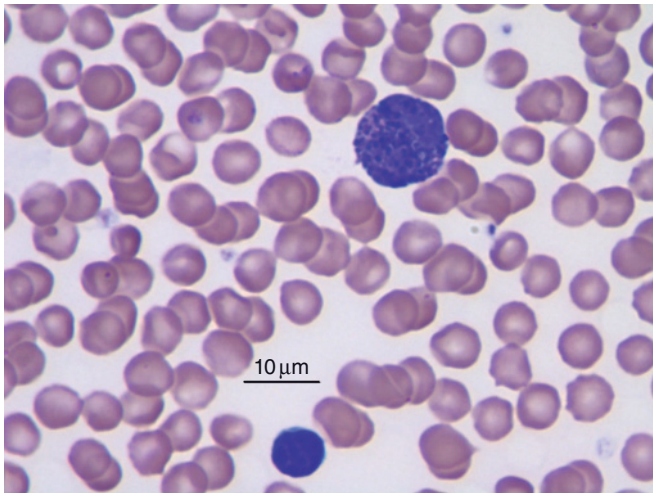


FIGURE 110.9 Erythrocytes with polychromasia, platelets, small lymphocyte, and basophil from a New Zealand White rabbit. (Wright's stain; 100× objective.)

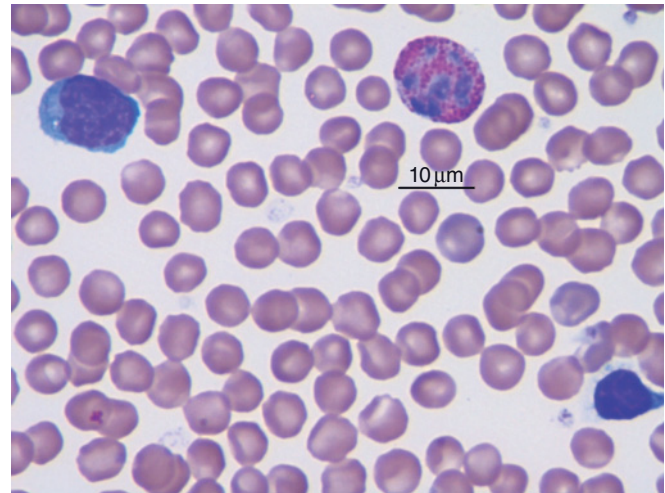


FIGURE 110.10 Erythrocytes with polychromasia, small and large lymphocyte, and heterophil from a New Zealand White rabbit. (Wright's stain; 100× objective.)

repeated blood collections, while gestation was associated with an initial increase then decline in reticulocyte count, Hct, and Hgb concentration.³⁸

The lifespan of rabbit RBCs ranges between 45 and 70 days, with an average lifespan of 57 days with a predicted random destruction rate of 0.5% per day.^{13,35} Red blood cell counts are low in newborn rabbits, returning to adult interval by 6 months of age, whereas the MCV and MCH values are higher than in adults.² Red blood cell count and Hgb concentration are slightly higher in male rabbits than in females (Table 110.5).

Platelets

Rabbit platelets appear as small clusters of azurophilic granules surrounded by pale blue cytoplasm.^{24,30} Observed reference intervals of circulating platelets vary considerably. These vary from tight ranges such as 110,000–206,000/ μL ¹⁶ to broad ranges like 200,000–1,000,000/ μL .⁷

Leukocytes

The WBC count in rabbits may vary with diurnal fluctuations, nutritional variation, and differences in age, gender, and breed. Additionally, the differential WBC count in a healthy rabbit fluctuates considerably when evaluated over a single month.²⁹ Total WBC counts were lowest in newborn and juvenile rabbits, reaching adult levels after 6–12 months of age (Table 110.6). The neutrophil:lymphocyte ratio (expressed as percentage) at 2 months of age was found to be 33:60 and changed to 45:45 after 12 months of age.¹³ However, in newborns the initial ratio was reversed at 58:31 and by 2 weeks had declined to approximately 31:59.

Rabbit neutrophils, referred to as heterophils, contain acidophilic granules. Some argue for the presence of

both neutrophils and heterophils in rabbits.²³ The rabbit heterophil is approximately 10–15 μm in diameter. Its polymorphic nucleus stains light purple with a light blue nuclear membrane. The nucleus is surrounded by a diffusely pink cytoplasm, which contains small acidophilic specific granules and variable numbers of large red granules (Fig. 110.10). The nucleus of the rabbit lymphocyte is round, condensed, and surrounded by a narrow band of blue-staining cytoplasm, which may occasionally contain azurophilic granules (in larger lymphocytes).³⁰ Both small and large forms of lymphocytes may be observed. The rabbit monocyte is a large cell (15–18 μm in diameter) with an ameboid nuclear pattern (lobulated, horseshoe- or bean-shaped) and diffuse and lightly stained nuclear chromatin, with the nucleus surrounded by blue cytoplasm that may contain a few vacuoles (Fig. 110.11).³⁰ The rabbit eosinophil is larger than the heterophil (12–16 μm diameter) and has a nucleus that is bilobed or horseshoe-shaped. Its intensely acidophilic cytoplasmic granules are more numerous and are three to four times the size of heterophil granules. Basophils are found in rabbit blood in small to moderate numbers.¹³ Basophil numbers are inversely proportional to the number of tissue mast cells.¹³ The nucleus of the rabbit basophils stains light purple, and the cytoplasm is packed with purple to black metachromic granules (Fig. 110.1).³⁰

Bone Marrow Evaluation and Cytology

Bone marrow samples may be obtained from an anesthetized rabbit from: (1) the wing of the ilium; (2) the proximal end of the femur; or (3) the proximal end of the humerus.³⁹ An 18-gauge Rosenthal pediatric biopsy needle can be used for bone marrow collection from the humerus and femur. The appearance of most cells and the maturation sequence in rabbit bone marrow is

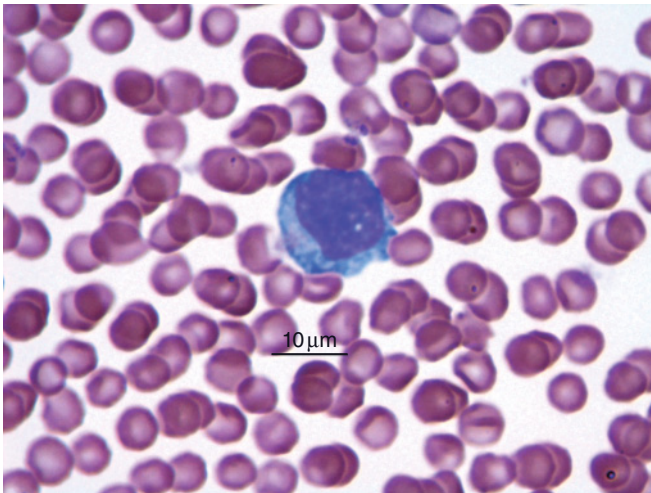


FIGURE 110.11 Erythrocytes with polychromasia, and monocyte from a New Zealand White rabbit. (Wright’s stain; 100× objective.)

TABLE 110.9 Differential Cell Distribution in the Bone Marrow of Normal Rabbits^a

Erythrocyte series	
Rubriblast	0.20%
Prorubricyte	0.60%
Basophilic rubricyte	5.50%
Orthochromatic rubricyte	18.90%
Metarubricyte	16.70%
Total erythroid cells	41.90%
Other cells	
Lymphocyte	12.60%
Monocyte	1.60%
Plasma cell	0.20%
RE cell	1.00%
Other	0.30%
Granulocytic series	
Myeloblast	0.70%
Progranulocyte	0.60%
Myelocyte	3.10%
Metamyelocyte	7.40%
Band Heterophil	23.20%
Segmenter Heterophil	5.30%
Basophil	0.70%
Eosinophil	1.40%
Total granulocytic cells	42.40%
M:E = 1:1	

^aRef. 3.

similar to that in humans and other mammals.²⁵ Sanderson and Phillips have provided an excellent series of color photomicrographs of rabbit bone marrow.³² The myeloid to erythroid (M:E) ratio of rabbit bone marrow is approximately 1:1.^{3,32} The M:E ratio in rabbits from birth to 5 months of age was reported as follows: 0.72:1 at birth, 0.19:1 at 1 week, 1.09:1 at 4 weeks, 0.61:1 at 2 months, 0.81:1 at 3 months, 1.42:1 at 4 months, and 0.89:1 at 5 months.^{3,31} Table 110.9 provides the differential cell counts of cells for bone marrow of healthy rabbits.

TABLE 110.10 Observed Coagulation Values in Rabbits

Parameter	Mean ± SD	Reference
Bleeding time (minutes)	1.4 ± 0.3	22
	2.1 ± 0.5	36
	1.9 ± 0.8	15
	5.4 ± 1.2	1
	4.6 ± 0.5	17
	3.87 ± 0.41	21
Clotting time (minutes)	4.3 ± 0.6	22
	4.0 ± 0.4	23
	4.0 ± 1.7	21
Prothrombin time (seconds)	7.5 ± 1.5	21
	7.5 ± 0.3	19
Activated partial thromboplastin time (seconds)	32.8 ± 4.5	21
	(19.5–22.5)	5
	(15.7–42.7)	26

Other Hematologic Values

Coagulation values for New Zealand White rabbits are provided in Table 110.10.^{21,22,24,25,36} The RBC sedimentation rate for blood from rabbits was reported as follows: for male rabbits, 2.0 ± 0.5 mm/hr (range, 1–3 mm/hr); for female rabbits, 1.75 ± 0.4 mm/hr (range, 0.95–2.55 mm/hr) and 2–4 mm/hr.^{28,31}

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HEMATOLOGY OF LABORATORY PRIMATES

KIRSTIN BARNHART

Blood Collection

Blood from non-human primates (NHPs) may be collected from a number of venipuncture sites depending on the size of the animal, the method of restraint, and the sample volume required. The most common site for the collection of large volumes is the femoral vein. The cephalic, saphenous, coccygeal, and jugular veins also can be used. For small samples, a sterile lancet may be used to puncture the marginal ear vein, a finger, or a heel. The average circulating blood volume of rhesus macaques, cynomolgus macaques, and marmosets has been estimated at 56, 65, and 70 mL/kg, respectively. In general, 7.5–20% of the blood volume can be removed in a 24 hour period without causing significant disturbance to the animal's normal physiology. The approximate recovery period ranges from 1 to 3 weeks depending on the amount removed.⁸

Blood collection from NHPs frequently requires chemical restraint. The most commonly used anesthetic agent is ketamine hydrochloride,³⁰ but other agents such as Telazol®, isoflurane and sevoflurane are frequently used. A physiologic leukocytosis typically occurs when blood is collected from non-anesthetized and untrained macaques. This leukocytosis is generally characterized by a lymphocytosis, but a mature neutrophilia may also occur.

Ketamine anesthesia reportedly reduces lymphocytes and the MCV, but increases the RBC count, Hgb concentration and MCHC in macaques of both sexes.³⁰ Conversely, other studies concluded that ketamine may reduce both WBC counts and RBC indices.² Sevoflurane may reduce WBC counts and RBC indices.⁵² However, others reported no hematologic effects of anesthetic agents including ketamine, pentobarbital, isoflurane, propofol, or Telazol® (Table 110.11).²²

The origin or strain of a particular NHP species can have significant effects on RBC and WBC indices. One of the most notable examples is the variation of hematologic parameters between cynomolgus monkeys that originate from Mauritius and those from South-East Asia. Specific differences include lower reticulocyte counts, higher RBC counts, decreased MCV, decreased MCH, and lower WBC counts in Mauritian monkeys.⁹ These differences are probably due to the isolated geography and lower degree of genetic variability in monkeys of Mauritian origin. Platelet counts and coagulation parameters are similar in monkeys from both locations.

Reference Intervals

Reference intervals reported in the literature are provided in Tables 110.11–110.22 for species including rhesus macaque, cynomolgus macaque, marmoset, tamarin, baboon, owl monkey, squirrel monkey, African green monkey and chimpanzee. These values may provide general guidelines and information for interpreting NHP hematology; however, it must be emphasized that laboratory methodologies and animal care procedures have changed substantially over the past 20 years, and caution should be used in comparing results with those in the literature. In addition to analytical methodology, many pre-analytical variables exist that have the potential to affect the accuracy of reference intervals. These variables include number of animals, the subpopulation of NHP species utilized, age, apparent health status, seasonal variations, diurnal variations, diet, environment and type of restraint.¹² The statistical method selected to describe a reference interval can also significantly affect the results. A majority of studies report reference intervals as the mean \pm one or two standard deviations. Depending on the number of animals utilized and the likelihood of Gaussian distribution, this may not be the most accurate method.

TABLE 110.11 Comparison of Erythrocyte and Leukocyte Parameters from Samples Obtained With and Without Anesthesia (Mean \pm SD)

Reference	Year	Strain	Sex	Age	No.	Select Condition	RBC ($\times 10^6/\mu\text{L}$)
30	2005	Cynomolgus Chinese	M	3–5 yrs	19 ^a	No anesthesia	5.26 \pm 0.34
			M	3–5 yrs	19 ^a	Ketamine	5.56 \pm 0.34
			F	3–5 yrs	16 ^a	No anesthesia	5.13 \pm 0.31
			F	3–5 yrs	16 ^a	Ketamine	5.35 \pm 0.39
22	1999	Rhesus	M	4–7 yrs	53 ^b	Ketamine	5.32 \pm 0.26
				4–7 yrs	54 ^b	Pentobarbital	5.43 \pm 0.44
				4–7 yrs	8 ^b	Isoflurane	5.07 \pm 0.31
58	1997	Rhesus	M/F	3 yrs	9/9 ^c	Ketamine	5.16 \pm 0.13
				3 yrs	9/9 ^c	Ketamine–Ace	5.26 \pm 0.14
				3 yrs	9/9 ^c	Telazol	5.17 \pm 0.11
2	1992	Rhesus	F	11–22 yrs	16 ^{d,e}	Ketamine	5.50 \pm 0.18
			F	11–22 yrs	16 ^{d,e}	Pre-ketamine	5.97 \pm 0.16

Reference	Year	Species	Sex	Age	No.	Select Condition	WBC ($\times 10^3/\mu\text{L}$)
30	2005	Cynomolgus Chinese	M	3–5 yrs	19 ^a	No anesthesia	13.1 \pm 3.3
			M	3–5 yrs	19 ^a	Ketamine	9.2 \pm 2.7
			F	3–5 yrs	16 ^a	No anesthesia	11.1 \pm 2.2
			F	3–5 yrs	16 ^a	Ketamine	8.5 \pm 3.6
22	1999	Rhesus	M	4–7 yrs	53 ^b	Ketamine	6.2 \pm 2.0
				4–7 yrs	54 ^b	Pentobarbital	5.8 \pm 2.3
				4–7 yrs	8 ^b	Isoflurane	5.5 \pm 2.3
58	1997	Rhesus	M/F	3 yrs	9/9 ^c	Ketamine	10.76 \pm 1.03
				3 yrs	9/9 ^c	Ketamine–Ace	10.11 \pm 0.96
				3 yrs	9/9 ^c	Telazol	9.65 \pm 0.67
2	1992	Rhesus	F	11–22 yrs	16 ^{d,e}	Ketamine	5.3 \pm 0.32
			F	11–22 yrs	16 ^{d,e}	Pre-ketamine	6.9 \pm 0.37

^aADVIA 120.^bBayer H1E blood analyzer.^cCoulter Corp^dSysmex E-2500: Toa Medical Electronics^eExpressed as mean \pm SEM

Erythrocytes

Red blood cells from NHPs are non-nucleated, biconcave discs with central pallor that are similar to those of other mammals. Some species, such as macaques, have a large area of central pallor that is reflected by lower MCHC values. A high degree of variability exists for RBC diameter and MCV due to the large number of species, many with marked phenotypic differences. Macaque RBC diameter ranges from 6.5 to 7.5 μm , and the range of RBC diameters across species varies between 3.5 and 8.5 μm .⁴⁷ Similar to humans, the circulating lifespan of macaque RBCs is approximately 100 days, and the normal reticulocyte counts are relatively low (0–80,000/ μL). The Hgb concentration, RBC count, PCV, MCV and MCHC are high for neonatal baboons, similar to human infants. These values begin to decline after the first week of life.³ Similarly, PCV, MCV and Hgb dropped significantly from 1–3 months after birth in healthy cynomolgus infants.⁵⁵ These findings are transitional and considered normal physiologic responses. After sexual maturity, male cynomolgus

monkeys, rhesus monkeys, baboons, chimpanzees and African green monkeys are frequently reported as having a higher MCV.^{12,15,16,26,46,54} The MCV and MCH for rhesus monkeys are similar to those of humans, but cynomolgus monkeys have lower values.

Heinz bodies have been described frequently in blood smears from healthy marmosets and occasionally from healthy tamarins.^{19,20} The clinical significance of these inclusions has been debated. The number of RBCs containing Heinz bodies ranged from 0% to 70%, and it is rare to find more than one inclusion per cell. Heinz bodies on marmoset RBCs do not adversely affect the properties of the membrane. Despite these findings, Heinz body hemolytic anemia has caused significant morbidity and mortality in marmoset colonies and has been linked to a frequently fatal disease termed Wasting Marmoset Syndrome.¹⁹ To date, Wasting Marmoset Syndrome is considered a form of inflammatory bowel disease that may result from many causes.⁵³ Similar to felines, marmoset RBCs may be more susceptible to Heinz body formation during times of stress and disease. Vitamin E supplementation has been

TABLE 110.11 Comparison of Erythrocyte and Leukocyte Parameters from Samples Obtained With and Without Anesthesia (Mean \pm SD) (continued)

Hgb (g/dL)	Hct (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	Platelets ($\times 10^3/\mu\text{L}$)	MPV (fL)	Reticulocytes (%)
12.7 \pm 0.8	47.5 \pm 2.6	90.3 \pm 3.2	24.0 \pm 0.7	26.6 \pm 0.8	421 \pm 82	—	1.7 \pm 0.4
13.6 \pm 0.8	47.0 \pm 2.4	84.7 \pm 2.8	24.5 \pm 0.8	28.9 \pm 0.9	431 \pm 92	—	2.0 \pm 0.4
12.2 \pm 0.8	47.0 \pm 2.4	91.7 \pm 3.3	23.9 \pm 1.2	26.0 \pm 0.8	445 \pm 71	—	1.6 \pm 0.5
12.9 \pm 1.1	45.4 \pm 3.9	84.8 \pm 3.7	24.0 \pm 1.0	28.3 \pm 0.7	462 \pm 60	—	1.7 \pm 0.4
12.6 \pm 0.5	38.9 \pm 1.6	73.1 \pm 2.1	23.7 \pm 0.9	32.2 \pm 1.2	310 \pm 50	—	—
12.7 \pm 0.9	39.4 \pm 1.9	73.2 \pm 2.7	23.7 \pm 0.8	31.9 \pm 1.1	304 \pm 47	—	—
12.0 \pm 0.6	36.2 \pm 1.9	71.4 \pm 2.3	23.7 \pm 0.9	33.3 \pm 0.4	350 \pm 62	—	—
11.5 \pm 0.2	36.4 \pm 0.7	70.7 \pm 0.9	22.3 \pm 0.3	31.6 \pm 0.3	351 \pm 26	7.9 \pm 0.2	—
11.5 \pm 0.2	37.1 \pm 0.7	70.9 \pm 0.9	22.0 \pm 0.3	31.1 \pm 0.2	346 \pm 20	7.8 \pm 0.3	—
11.4 \pm 0.2	36.7 \pm 0.6	71.4 \pm 0.9	22.2 \pm 0.3	31.2 \pm 0.2	364 \pm 22	7.6 \pm 0.3	—
13.0 \pm 0.4	41.1 \pm 1.3	74.8 \pm 0.7	23.5 \pm 0.2	31.4 \pm 0.2	475 \pm 37	—	1.7 \pm 0.2
14.1 \pm 10.4	44.7 \pm 1.0	75.0 \pm 0.8	23.6 \pm 0.2	31.4 \pm 0.2	490 \pm 50	—	1.5 \pm 0.1

Neutrophils ($\times 10^3/\mu\text{L}$)	Lymphocytes ($\times 10^3/\mu\text{L}$)	Monocytes ($\times 10^3/\mu\text{L}$)	Eosinophils ($\times 10^3/\mu\text{L}$)	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)	Basophils (%)
—	—	—	—	26.0 \pm 10.0	67.3 \pm 9.5	3.5 \pm 0.9	0.9 \pm 0.4	4.3 \pm 3.2
—	—	—	—	36.1 \pm 11.0	59.0 \pm 10.6	3.0 \pm 1.0	0.7 \pm 0.6	0.8 \pm 1.0
—	—	—	—	28.8 \pm 13.8	63.3 \pm 13.4	3.8 \pm 1.4	1.6 \pm 0.8	2.9 \pm 1.6
—	—	—	—	38.4 \pm 13.0	55.0 \pm 12.7	3.3 \pm 1.0	2.1 \pm 1.4	0.4 \pm 0.2
—	—	—	—	39.5 \pm 12.9	55.4 \pm 16.2	2.9 \pm 1.4	2.2 \pm 1.7	0.2 \pm 0.2
—	—	—	—	41.2 \pm 11.5	51.5 \pm 11.5	3.8 \pm 1.9	2.2 \pm 1.9	0.4 \pm 1.1
—	—	—	—	33.2 \pm 11.5	63.3 \pm 11.0	1.4 \pm 1.9	1.6 \pm 1.7	0.1 \pm 0.3
—	—	—	—	47.6 \pm 5.0	46.1 \pm 4.7	2.7 \pm 0.4	2.7 \pm 0.5	0.3 \pm 0.1
—	—	—	—	45.1 \pm 4.5	49.1 \pm 4.4	2.7 \pm 0.4	2.0 \pm 0.3	0.3 \pm 0.1
—	—	—	—	44.9 \pm 4.0	48.8 \pm 4.0	2.8 \pm 0.4	2.2 \pm 0.4	0.4 \pm 0.1
3.03 \pm 0.27	1.92 \pm 0.15	0.14 \pm 0.03	0.25 \pm 0.06	—	—	—	—	—
2.60 \pm 0.21	3.90 \pm 0.37	0.17 \pm 0.03	0.36 \pm 0.08	—	—	—	—	—

reported to decrease the incidence of Heinz body anemia.

Low numbers of nucleated RBCs, typically 1–2 per 100 WBCs, are frequently seen in healthy New World monkeys (marmosets, tamarins, owl monkeys, squirrel monkeys) and probably reflect the relatively high RBC turnover rate of these species.^{19,20,37} Slight to mild anisocytosis, polychromasia, and Howell-Jolly bodies are also commonly described for owl monkeys, squirrel monkeys, and tamarins.^{7,19,20,37} Female tamarins typically have a lower PCV, Hgb, and RBC count and a higher reticulocyte count compared to males.^{20,21} Poikilocytes and three types of reticulocytes are found frequently in healthy colony-born owl monkeys.³⁷ Low numbers of target cells (<10%) are commonly identified in blood smears from chimpanzees.

Stomatocytosis is a frequent finding in blood smears from normal squirrel and owl monkeys. The cause of the stomatocytosis is not known, and the number of stomatocytes in blood varies. A study performed on a colony of seven captive squirrel monkeys identified 9.4–89.2% stomatocytes (average 52%).¹⁰ In addition,

this study documented an association between increased numbers of stomatocytes and elevated Hcts that was probably caused by alterations in the normal packing characteristics of RBCs. Unlike many inherited and acquired forms of stomatocytosis in other species, squirrel monkey RBCs do not show increased osmotic fragility and shortened survival.

Old World monkeys and great apes have a true menstrual cycle, which can affect interpretation of RBC indices because a significant amount of blood may be lost during menstruation.¹³ In addition, baboons and chimpanzees display marked turgescence and detumescence of the sex skin in association with the follicular and luteal phases of the menstrual cycle, respectively. During turgescence, plasma volume contracts, and as much as 2 liters of extracellular fluid can accumulate in the perineum. In early detumescence, this fluid is rapidly auto-infused into the plasma. These fluid shifts can affect RBC indices.^{14,17} A study by Harewood et al. described a decrease in Hgb concentration, Hct, RBC count and WBC count, and an increased platelet count in the luteal phase as compared to the follicular phase.¹⁷

TABLE 110.12 Selected Erythrocyte and Leukocyte Parameters for the Common Marmoset (*Callithrix jacchus*) (Mean \pm SD or Range)

Reference	Year	Sex	Age	No.	RBC ($\times 10^6/\mu\text{L}$)	Hgb (g/dL)	Hct (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	Platelets ($\times 10^3/\mu\text{L}$)	MPV	Reticulocytes (%)
59	1984	M	Adult	55–61 ^a	5.65 \pm 1.00	16.1 \pm 3.5	42.7 \pm 11.2	72 \pm 6	28.0 \pm 4.2	39.0 \pm 5.5	281 \pm 101	—	2.4 \pm 2.3
		F	Adult	33–38 ^a	5.74 \pm 1.10	15.0 \pm 1.8	41.3 \pm 5.0	73 \pm 4	26.9 \pm 3.7	37.0 \pm 3.8	281 \pm 121	—	1.9 \pm 1.7
		M	Juv	41–46 ^a	5.07 \pm 0.78	13.9 \pm 1.3	35.8 \pm 5.8	71 \pm 5	29.0 \pm 5.0	41.1 \pm 7.3	344 \pm 154	—	2.8 \pm 2.4
		F	Juv	28–32 ^a	5.04 \pm 0.65	13.6 \pm 1.3	36.9 \pm 5.2	71 \pm 7	27.4 \pm 3.1	37.6 \pm 5.1	328 \pm 123	—	4.4 \pm 4.0
19	1982	M/F	<1 yr	13 ^{a,b}	5.5–6.9	12.0–16.0	39–47	59.9–79.1	19.3–26.1	30.5–34.5	NA	—	0.4–5.9
		M/F	Adult	30 ^{a,b}	6.1–7.7	13.3–17.7	42–55	62.7–75.9	19.8–25.0	30.3–34.3	298–682	—	0.9–7.7

Reference	Year	Sex	Age	No.	WBC ($\times 10^3/\mu\text{L}$)	Neutrophils ($\times 10^3/\mu\text{L}$)	Lymphocytes ($\times 10^3/\mu\text{L}$)	Monocytes ($\times 10^3/\mu\text{L}$)	Eosinophils ($\times 10^3/\mu\text{L}$)	Basophils ($\times 10^3/\mu\text{L}$)	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)	Basophils (%)
59	1984	M	Adult	55–61 ^a	8.1 \pm 3.2	—	—	—	—	—	43 \pm 16	51 \pm 16	3.3 \pm 2.9	0.4 \pm 1.0	0.8 \pm 1.3
		F	Adult	33–38 ^a	7.4 \pm 2.8	—	—	—	—	—	54 \pm 15	40 \pm 14	3.6 \pm 2.8	0.5 \pm 0.9	1.5 \pm 2.1
		M	Juv	41–46 ^a	9.3 \pm 3.7	—	—	—	—	—	46 \pm 14	50 \pm 16	1.1 \pm 1.2	0.6 \pm 1.0	0.8 \pm 1.2
		F	Juv	28–32 ^a	9.1 \pm 3.4	—	—	—	—	—	47 \pm 17	51 \pm 17	1.0 \pm 1.0	0.3 \pm 0.7	1.0 \pm 0.9
19	1982	M/F	<1 yr	13 ^{a,b}	5.9–8.3	0.9–4.1	2.5–6.1	0–0.4	0–0.3	0–0.3	13–59	36–87	0–6	0–4	0–4
		M/F	Adult	30 ^{a,b}	4.1–10.5	1.0–7.0	1.0–5.0	0–0.3	0–0.3	0–0.5	26–84	16–70	0–3	0–6	0–8

^aCoulter Counter.^bExpressed as mean \pm 2SD.

TABLE 110.13 Selected Erythrocyte and Leukocyte Parameters for Tamarins (Range)

Reference	Year	Species	Sex	Age	No.	RBC ($\times 10^6/\mu\text{L}$)	Hgb (g/dL)	Hct (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	Platelets ($\times 10^3/\mu\text{L}$)	Reticulocytes (%)
21	1983	<i>Sagunius labiatus</i> (Red-bellied tamarin)	M/F	Adult	25 ^a	5.5–8.4	13.6–20.5	43–58	65.2–80.6	22.4–26.0	31.7–35.7	296–564	—
20	1983	<i>Saguinus oedipus</i> (Cotton-top tamarin)	M	Adult	25 ^{b,c}	5.7–7.7	15.5–20.1	50–58	70.0–83.2	23.8–28.7	31.9–36.8	195–607	0.7–6.2
			F	Adult	18 ^c	5.4–7.2	13.3–18.8	30–53	70.0–83.2	22.2–28.1	31.8–35.0	195–607	0.4–11.5

Reference	Year	Species	Sex	Age	No.	WBC ($\times 10^3/\mu\text{L}$)	Neutrophils ($\times 10^3/\mu\text{L}$)	Lymphocytes ($\times 10^3/\mu\text{L}$)	Monocytes ($\times 10^3/\mu\text{L}$)	Eosinophils ($\times 10^3/\mu\text{L}$)	Basophils ($\times 10^3/\mu\text{L}$)	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)	Basophils (%)
21	1983	<i>Sagunius labiatus</i> (Red-bellied tamarin)	M/F	Adult	25 ^a	4.5–11.9	0.42–8.14	1.4–5.44	0–0.77	0–0.47	0–0.38	28–84	12–61	0–9	0–5	0–4
20	1983	<i>Saguinus oedipus</i> (Cotton-top tamarin)	M	Adult	25 ^{b,c}	4.2–11.1	2.10–8.68	0.91–4.28	0–0.34	0–0.30	0–0.26	53–78	10–56	0–5	0–0	0–4
			F	Adult	18 ^c	4.2–11.1	2.10–8.68	0.91–4.28	0–0.34	0–0.30	0–0.26	53–78	15–55	0–5	0–0	0–4

^aInterval represents mean \pm 2 SD.^bIf a t test detected no difference between the mean \pm 2SD for each sex, the interval was considered the same for both.^cCoulter counter.

TABLE 110.14 Selected Erythrocyte and Leukocyte Parameters for Squirrel Monkeys (Range and/or Mean \pm SD)

Reference	Year	Species	Sex	Age	No.	RBC ($\times 10^6/\mu\text{L}$)	Hgb (g/dL)	Hct (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	RDW (%)	Platelets ($\times 10^3/\mu\text{L}$)	MPV	Reticulocytes (%)
28	1987	<i>Saimiri boliviensis boliviensis</i>	M	Adult	38	6.46–8.09	12.8–15.5	39.7–50.6	58.0–65.3	18.2–21.1	30.3–34.6	—	277–911	11.9–22.4	—
27	1985	<i>Saimiri sciureus</i> (Bolivian) – wild	M	Adult	14 ^{a,b}	7.47 \pm 0.1 (6.59–8.09)	14.6 \pm 0.16 (13.7–15.5)	46.3 \pm 0.64 (41.8–49.5)	62.0 \pm 0.53 (58.0–65.3)	19.6 \pm 0.19 (18.2–20.5)	31.6 \pm 0.18 (30.3–32.8)	16.7 \pm 0.32 (15.5–19.6)	572 \pm 50 (473–899)	12.3 \pm 0.36 (10.2–14.4)	2.6 \pm 0.23 (1.5–3.9)
			M	Adult	9 ^{a,b}	7.12 \pm 0.1 (6.85–7.69)	13.8 \pm 0.18 (12.8–14.5)	44.0 \pm 0.64 (40.3–46.4)	61.9 \pm 0.64 (58.7–64.1)	19.4 \pm 0.19 (18.6–20.1)	31.3 \pm 0.23 (30.3–32.2)	17.4 \pm 0.49 (15.7–19.9)	683 \pm 39 (538–884)	12.5 \pm 0.49 (11.1–15.7)	3.1 \pm 3.4 (1.9–4.6)
7	1970	<i>Saimiri sciureus</i> (Bolivian) – lab	F	Adult	31	6.87 \pm 0.57 (5.7–8.12)	13.7 \pm 1.2 (11.6–17.1)	41.1 \pm 3.3 (35.0–51.5)	59.9 \pm 3.9 (48.6–66.7)	—	33.4 \pm 1.4 (31.4–37.3)	—	448 \pm 64 (378–561)	—	—

Reference	Year	Species	Sex	Age	No.	WBC ($\times 10^3/\mu\text{L}$)	Neut ($\times 10^3/\mu\text{L}$)	Lymphocytes ($\times 10^3/\mu\text{L}$)	Monocytes ($\times 10^3/\mu\text{L}$)	Eosinophils ($\times 10^3/\mu\text{L}$)	Basophils ($\times 10^3/\mu\text{L}$)	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)	Basophils (%)
28	1987	<i>Saimiri boliviensis boliviensis</i>	M	Adult	38	4.7–14.6	—	—	—	—	—	13–63	34–76	1–17	N/A	N/A
27	1985	<i>Saimiri sciureus</i> (Bolivian) – wild	M	Adult	14 ^{a,b}	7.6 \pm 0.34 (5.3–10.1)	—	—	—	—	—	41 \pm 4.0 (21–66)	54 \pm 4.0 (30–73)	2 \pm 0.3 (1–5)	1 \pm 0.2 (0–3)	1 \pm 0.2 (0–2)
			M	Adult	9 ^{a,b}	10.5 \pm 0.64 (7.8–13.5)	—	—	—	—	—	35 \pm 3.2 (20–54)	61 \pm 3.1 (42–75)	2 \pm 0.3 (1–3)	1 \pm 0.2 (0–2)	0 \pm 0.2 (0–1)
7	1970	<i>Saimiri sciureus</i> (Bolivian) – lab	F	Adult	31	11.2 \pm 5.2 (5.7–23.6)	6.68 \pm 4.62 (1.54–17.23)	4.02 \pm 1.08 (2.26–7.32)	0.23 \pm 0.34 (0–1.62)	0.59 \pm 0.51 (0–1.72)	0.01 \pm 0.03 (0–0.12)	52.7 \pm 16.8 (23–80)	38.9 \pm 11.3 (18–69)	2.0 \pm 1.8 (0–7)	6.2 \pm 6.1 (0–21)	0.1 \pm 0.3 (0–1)

^aCoulter counter, S-Plus II.

^bExpressed as mean \pm SEM

TABLE 110.15 Selected Erythrocyte and Leukocyte Parameters for Owl Monkeys (Range)

Reference	Year	Species	Sex	Age	No.	RBC ($\times 10^6/\mu\text{L}$)	Hgb (g/dL)	Hct (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	RDW (%)	Platelets ($\times 10^3/\mu\text{L}$)
35	1995	<i>Aotus vociferans</i>	M	Adult	18 ^a	5.6–7.4	14.7–18.9	44.0–57.0	72.0–88.0	22.7–28.9	31.4–33.8	—	111–480
			F	Adult	17 ^a	5.8–7.0	14.0–18.9	45.1–57.8	74.0–90.0	23.0–30.3	31.0–33.6	—	62–409
34	1990	<i>Aotus nancymae</i>	M	Adult	130 ^{a,b}	5.0–7.6	13.1–18.8	41.3–58.0	72.0–87.7	22.5–29.3	30.5–34.9	—	207–623
			F	Adult	124 ^{a,b}	4.4–7.3	12.0–19.7	35.8–57.5	72.3–87.0	22.6–29.3	30.6–34.4	—	157–690

Reference	Year	Species	Sex	Age	No.	WBC ($\times 10^3/\mu\text{L}$)	Neutrophils ($\times 10^3/\mu\text{L}$)	Lymphocytes ($\times 10^3/\mu\text{L}$)	Monocytes ($\times 10^3/\mu\text{L}$)	Eosinophils ($\times 10^3/\mu\text{L}$)	Basophils ($\times 10^3/\mu\text{L}$)	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)	Basophils (%)
35	1995	<i>Aotus vociferans</i>	M	Adult	18 ^a	5.3–18.5	0.90–5.60	2.40–8.10	0–0.70	0.4–3.00	0–0.20	—	—	—	—	—
			F	Adult	17 ^a	4.4–14.1	0.80–3.70	3.00–7.40	0–1.10	0.30–4.00	0–0.50	—	—	—	—	—
34	1990	<i>Aotus nancymae</i>	M	Adult	130 ^a	4.7–16.4	0.53–9.28	2.55–12.06	0–1.08	0–2.09	0–0.72	5.3–65.6	25.3–87.7	0–10.4	0–15.0	0–5.0
			F	Adult	124 ^a	4.2–17.6	0.15–7.29	2.28–14.68	0–1.19	0–1.74	0–0.65	3.0–58.8	26.9–91.9	0–9.9	0–15.9	0–7.2

^aOrtho ELT-8 automated analyzer.

^bRange expressed as the difference between the 2.5 and 97.5 percentiles.

TABLE 110.16 Selected Erythrocyte and Leukocyte Parameters for African Green Monkeys (Mean ± SD)

Reference	Year	Sex	Age	No.	RBC ($\times 10^6/\mu\text{L}$)	Hgb (g/dL)	Hct (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	RDW (%)	Platelets ($\times 10^3/\mu\text{L}$)	MPV (fL)
26	2007	M	Adult	10 ^a	6.5 ± 0.8	15.1 ± 1.2	50.6 ± 6.2	77.2 ± 3.1	24.0 ± 1.3	31.4 ± 1.4	13.9 ± 0.8	318 ± 76	7.2 ± 0.9
		F	Adult	13 ^a	5.4 ± 0.5	12.3 ± 1.2	40.3 ± 4.0	74.9 ± 3.9	22.9 ± 1.1	30.7 ± 0.9	14.1 ± 1.1	387 ± 85	6.8 ± 0.6
		M	Juv	13 ^a	5.3 ± 0.5	12.1 ± 1.2	40.6 ± 3.4	76.6 ± 1.9	22.6 ± 0.8	29.7 ± 0.6	14.0 ± 0.7	321 ± 78	7.5 ± 1.4
		F	Juv	14 ^a	5.2 ± 0.2	11.8 ± 0.5	39.7 ± 1.8	75.8 ± 3.5	22.4 ± 1.0	30.0 ± 0.8	13.9 ± 1.0	385 ± 81	6.7 ± 0.8
46	2005	F	1 yr	7 ^b	5.50 ± 0.21	12.7 ± 0.4	37.4 ± 1.0	67.8 ± 1.5	23.2 ± 0.6	34.1 ± 0.3	—	—	—
		M	1 yr	8 ^b	5.77 ± 0.30	12.8 ± 0.8	38.0 ± 2.4	65.7 ± 1.6	22.1 ± 0.7	33.6 ± 0.6	—	—	—
		F	2–3 yrs	14 ^b	5.67 ± 0.28	13.2 ± 0.8	38.6 ± 2.1	68.0 ± 2.0	23.3 ± 0.9	34.3 ± 0.6	—	—	—
		M	2–3 yrs	14 ^b	5.62 ± 0.54	13.0 ± 1.0	37.6 ± 3.0	67.1 ± 2.0	23.1 ± 0.9	34.4 ± 0.5	—	—	—
		F	4–6 yrs	7 ^b	5.31 ± 0.50	12.9 ± 1.0	36.8 ± 3.3	69.4 ± 1.0	24.4 ± 0.4	35.2 ± 0.4	—	—	—
		M	4–6 yrs	24 ^b	6.56 ± 0.53	15.5 ± 1.0	44.7 ± 3.2	68.2 ± 1.9	23.6 ± 0.7	34.6 ± 0.6	—	—	—
		F	7–10 yrs	16 ^b	5.77 ± 0.52	13.7 ± 1.1	39.4 ± 3.2	68.4 ± 2.8	23.8 ± 1.0	34.9 ± 0.4	—	—	—
		M	7–10 yrs	24 ^b	6.79 ± 0.76	16.2 ± 1.7	47.0 ± 5.1	69.4 ± 3.1	23.8 ± 1.3	34.4 ± 0.6	—	—	—
		F	11–14 yrs	10 ^b	5.48 ± 0.35	13.1 ± 0.7	37.4 ± 2.0	68.2 ± 1.6	23.8 ± 0.5	35.0 ± 0.3	—	—	—
		M	11–14 yrs	5 ^b	7.20 ± 0.27	16.9 ± 0.6	49.5 ± 2.0	68.8 ± 1.3	23.4 ± 0.5	34.2 ± 0.4	—	—	—
		F	15+ yrs	6 ^b	6.09 ± 0.61	14.4 ± 1.5	41.7 ± 4.1	68.3 ± 1.9	23.7 ± 1.1	34.6 ± 0.9	—	—	—
		M	15+ yrs	5 ^b	6.76 ± 0.63	15.8 ± 1.3	46.1 ± 3.4	68.4 ± 1.7	23.5 ± 0.5	34.4 ± 0.4	—	—	—
Reference	Year	Sex	Age	No.	WBC ($\times 10^3/\mu\text{L}$)	Neutrophils ($\times 10^3/\mu\text{L}$)	Lymphocytes ($\times 10^3/\mu\text{L}$)	Monocytes ($\times 10^3/\mu\text{L}$)	Eosinophils ($\times 10^3/\mu\text{L}$)	Basophils ($\times 10^3/\mu\text{L}$)			
26	2007	M	Adult	10 ^a	6.5 ± 2.3	2.5 ± 1.3	3.5 ± 1.9	0.6 ± 0.3	—	—			
		F	Adult	13 ^a	7.5 ± 1.9	3.6 ± 2.1	3.4 ± 1.5	0.6 ± 0.2	—	—			
		M	Juv	13 ^a	6.5 ± 1.5	1.7 ± 0.9	4.2 ± 1.3	0.6 ± 0.3	—	—			
		F	Juv	14 ^a	5.6 ± 1.1	1.9 ± 1.1	3.1 ± 0.6	0.7 ± 0.3	—	—			
46	2005	F	1 yr	7 ^b	6.5 ± 2.2	3.53 ± 1.83	3.26 ± 1.92	0.33 ± 0.01	0	0.02 ± 0.03			
		M	1 yr	8 ^b	6.4 ± 2.0	3.51 ± 2.52	2.53 ± 1.32	0.21 ± 0.12	0.01 ± 0.02	0.08 ± 0.06			
		F	2–3 yrs	14 ^b	7.0 ± 2.8	4.56 ± 2.92	2.18 ± 1.25	0.28 ± 0.17	0.04 ± 0.06	0.04 ± 0.04			
		M	2–3 yrs	14 ^b	6.2 ± 3.0	3.63 ± 3.02	2.19 ± 1.47	0.24 ± 0.08	0.02 ± 0.03	0.04 ± 0.04			
		F	4–6 yrs	7 ^b	7.3 ± 2.4	5.43 ± 2.94	1.67 ± 0.72	0.25 ± 0.08	0.02 ± 0.03	0.01 ± 0.03			
		M	4–6 yrs	24 ^b	7.5 ± 3.3	4.34 ± 2.42	2.30 ± 1.03	0.30 ± 0.19	0.02 ± 0.04	0.03 ± 0.03			
		F	7–10 yrs	16 ^b	6.8 ± 2.0	4.25 ± 2.10	2.19 ± 1.00	0.34 ± 0.14	0.02 ± 0.04	0.04 ± 0.04			
		M	7–10 yrs	24 ^b	7.3 ± 2.6	3.89 ± 1.78	2.74 ± 0.96	0.35 ± 0.13	0.03 ± 0.04	0.05 ± 0.04			
		F	11–14 yrs	10 ^b	8.5 ± 3.5	5.79 ± 3.60	2.32 ± 0.88	0.44 ± 0.20	0.04 ± 0.05	0.04 ± 0.05			
		M	11–14 yrs	5 ^b	5.7 ± 1.4	2.71 ± 0.67	2.67 ± 0.86	0.25 ± 0.09	0.01 ± 0.02	0.05 ± 0.05			
F	15+ yrs	6 ^b	8.0 ± 3.1	5.92 ± 3.40	1.63 ± 1.04	0.36 ± 0.12	0.01 ± 0.02	0.05 ± 0.05					
M	15+ yrs	5 ^b	6.3 ± 1.3	2.70 ± 0.39	3.19 ± 1.19	0.35 ± 0.10	0.05 ± 0.05	0.01 ± 0.02					

^aCoulter A^c.T diff.^bCell-Dyn 3500.

TABLE 110.17 Selected Erythrocyte and Leukocyte Parameters for Chimpanzees (Mean \pm SD and/or Range)

Reference	Year	Sex	Age	No.	RBC					Platelets ($\times 10^3/\mu\text{L}$)	
					($\times 10^6/\mu\text{L}$)	Hgb (g/dL)	Hct (%)	MCV (fL)	MCH (pg)		MCHC (g/dL)
23	2003	M	Infant (0–4 yrs)	15–18 ^a	5.1 \pm 0.9 (4.2–6.0)	12.5 \pm 2.4 (10.1–14.9)	39.0 \pm 5.7 (33.3–44.7)	76.4 \pm 11.6 (64.8–88.0)	24.6 \pm 4.5 (20.1–29.1)	32.2 \pm 2.2 (30.0–34.4)	309 \pm 149 (160–458)
		M	Juvenile (4–7 yrs)	14–23 ^a	5.0 \pm 0.6 (4.4–5.6)	13.2 \pm 1.4 (11.9–14.6)	40.4 \pm 4.1 (36.3–44.5)	80.7 \pm 5.8 (74.9–86.4)	26.2 \pm 4.5 (21.8–30.7)	32.8 \pm 1.5 (31.2–34.3)	282 \pm 110 (172–391)
		M	Adolescent (7–10 yrs)	18–28 ^a	5.3 \pm 0.8 (4.5–6.1)	14.4 \pm 2.0 (12.4–16.4)	43.8 \pm 5.9 (37.9–49.7)	82.9 \pm 6.8 (76.1–89.7)	27.3 \pm 2.7 (24.6–30.0)	32.9 \pm 1.8 (31.1–34.7)	264 \pm 133 (131–398)
		M	Adult (>10 yrs)	25–26 ^a	5.5 \pm 0.7 (4.8–6.2)	15.4 \pm 1.5 (13.9–16.9)	47.2 \pm 4.8 (42.4–52.0)	86.1 \pm 6.7 (79.4–92.9)	28.1 \pm 2.7 (25.5–30.8)	32.7 \pm 1.8 (30.9–34.4)	196 \pm 96 (99–292)
		F	Infant (0–4 yrs)	10–20 ^a	5.1 \pm 0.8 (4.4–5.9)	13.0 \pm 1.5 (11.5–14.4)	39.8 \pm 4.5 (35.3–44.3)	78.0 \pm 7.4 (70.6–85.3)	25.5 \pm 2.5 (22.1–29.0)	32.6 \pm 1.8 (30.9–34.4)	323 \pm 173 (151–496)
		F	Juvenile (4–7 yrs)	16–29 ^a	4.9 \pm 1.0 (4.0–5.9)	13.1 \pm 1.9 (11.2–15.0)	40.2 \pm 5.7 (34.5–45.9)	80.9 \pm 6.9 (74.0–87.9)	26.3 \pm 2.6 (23.7–28.9)	32.5 \pm 2.0 (30.6–34.5)	307 \pm 135 (172–442)
		F	Adolescent (7–10 yrs)	21–27 ^a	4.9 \pm 0.7 (4.2–5.5)	13.1 \pm 1.6 (11.6–14.7)	40.1 \pm 4.7 (35.5–44.8)	82.3 \pm 6.5 (75.8–88.8)	26.9 \pm 2.9 (24.1–29.8)	32.7 \pm 2.0 (30.7–34.7)	281 \pm 106 (174–387)
		F	Adult (>10 yrs)	37 ^a	5.1 \pm 0.9 (4.2–6.0)	13.6 \pm 2.1 (11.5–15.7)	42.0 \pm 6.6 (35.4–48.6)	83.1 \pm 6.9 (76.2–90)	26.9 \pm 2.6 (24.3–29.5)	32.4 \pm 2.1 (30.3–34.5)	230 \pm 133 (97–363)
24	2001	M	Infant (0–3 yrs)	18–36 ^{b,c}	4.71–5.73	11.5–14.5	34.7–43.1	70.1–80.8	23.2–26.6	32.5–34.0	202–557
		M	Juvenile (3–6 yrs)	22–32 ^{b,c}	4.53–5.51	11.9–15.2	35.7–46.1	74.9–82.2	23.9–27.6	31.9–34.2	247–487
		M	Adolescent (6–10 yrs)	17–27 ^{b,c}	4.59–5.97	13.0–16.9	37.3–49.7	76.5–84.7	25.3–28.7	32.5–35.2	84–385
		M	Adult (>10 yrs)	27 ^{b,c}	4.99–6.36	13.2–17.3	40.5–51.0	76.4–88.6	24.7–29.6	32.3–33.8	131–380
		F	Infant (0–3 yrs)	18–38 ^{b,c}	4.70–6.16	11.6–14.9	35.7–44.7	66.7–80.6	21.9–28.6	29.9–34.4	160–570
		F	Juvenile (3–6 yrs)	19–35 ^{b,c}	4.57–5.33	12.1–14.6	36.5–43.6	74.7–84.0	24.7–27.7	32.0–34.4	225–413
		F	Adolescent (6–10 yrs)	17–33 ^{b,c}	4.63–5.52	12.8–15.5	37.5–45.6	74.5–86.5	25.0–29.1	32.4–34.3	184–365
		F	Adult (>10 yrs)	48–49 ^{b,c}	4.65–5.78	12.3–15.3	37.6–45.8	76.0–87.7	24.4–29.6	32.1–34.2	151–324

Reference	Year	Sex	Age	No.	WBC					
					($\times 10^3/\mu\text{L}$)	Neutrophils ($\times 10^3/\mu\text{L}$)	Lymphocytes ($\times 10^3/\mu\text{L}$)	Monocytes ($\times 10^3/\mu\text{L}$)	Eosinophils ($\times 10^3/\mu\text{L}$)	Basophils ($\times 10^3/\mu\text{L}$)
23	2003	M	Infant (0–4 yrs)	15–18 ^a	9.7 \pm 5.6 (4.1–15.3)	3.2 \pm 2.9 (0.3–6.2)	5.2 \pm 3.5 (1.7–8.7)	0.4 \pm 0.3 (0.1–0.6)	0.2 \pm 0.3 (0–0.5)	0.1 (0–0.1)
		M	Juvenile (4–7 yrs)	14–23 ^a	9.4 \pm 7.4 (2.0–16.8)	4.6 \pm 6.3 (1.3–10.9)	3.9 \pm 3.8 (1.4–7.7)	0.4 \pm 0.4 (0.1–0.8)	0.2 \pm 0.4 (0–0.6)	0 (0–0.1)
		M	Adolescent (7–10 yrs)	18–28 ^a	8.9 \pm 5.4 (3.4–14.3)	4.7 \pm 5.5 (1.0–10.2)	3.3 \pm 2.1 (1.2–5.5)	0.3 \pm 0.4 (0–0.7)	0.2 \pm 0.2 (0–0.4)	0 (0–0.1)
		M	Adult (>10 yrs)	25–26 ^a	9.2 \pm 6.9 (2.3–16.1)	5.4 \pm 5.8 (1.5–11.2)	2.6 \pm 1.8 (0.8–4.4)	0.3 \pm 0.4 (0–0.7)	0.1 \pm 0.3 (0–0.4)	0 (0–0.1)
		F	Infant (0–4 yrs)	10–20 ^a	10.7 \pm 10.5 (4.1–21.2)	3.8 \pm 5.0 (0.9–8.8)	5.5 \pm 3.0 (2.5–8.6)	0.3 \pm 0.5 (0.1–0.8)	0.3 \pm 0.8 (0–1.1)	0.1 (0–0.1)
		F	Juvenile (4–7 yrs)	16–29 ^a	10.0 \pm 6.7 (3.3–16.7)	4.8 \pm 6.9 (1.4–11.7)	4.0 \pm 2.8 (1.2–6.8)	0.4 \pm 0.3 (0–0.7)	0.2 \pm 0.3 (0–0.5)	0.1 (0–0.1)
		F	Adolescent (7–10 yrs)	21–27 ^a	10.4 \pm 7.3 (3.1–17.7)	5.3 \pm 6.1 (2.0–11.4)	4.2 \pm 5.3 (2.0–9.5)	0.4 \pm 0.4 (0.1–0.8)	0.3 \pm 0.5 (0–0.8)	0 (0–0.1)
		F	Adult (>10 yrs)	37 ^a	9.1 \pm 6.3 (2.9–15.4)	4.5 \pm 8.5 (0–13.1)	3.7 \pm 3.1 (0.6–6.8)	0.4 \pm 0.5 (0–0.9)	0.3 \pm 0.6 (0–0.8)	0.1 (0–0.2)
24	2001	M	Infant (0–3 yrs)	18–36 ^{b,c}	6.34–16.26	2.00–10.21	3.15–7.92	0.02–0.59	0–0.55	0–0
		M	Juvenile (3–6 yrs)	22–32 ^{b,c}	7.23–16.38	2.88–10.93	2.02–5.74	0.01–0.53	0–0.40	0–0
		M	Adolescent (6–10 yrs)	17–27 ^{b,c}	5.71–15.72	2.67–10.49	1.84–7.09	0–0.55	0.07–0.51	0–0.02
		M	Adult (>10 yrs)	27 ^{b,c}	8.24–15.67	4.54–10.67	2.01–6.13	0.16–0.57	0.02–0.31	0–0.02
		F	Infant (0–3 yrs)	18–38 ^{b,c}	7.42–15.51	1.82–7.71	3.79–10.15	0–0.56	0.05–0.85	0–0.03
		F	Juvenile (3–6 yrs)	19–35 ^{b,c}	6.77–16.13	2.48–11.41	2.31–7.20	0.07–0.56	0.01–0.58	0–0.03
		F	Adolescent (6–10 yrs)	17–33 ^{b,c}	6.61–16.01	3.33–11.68	2.53–8.19	0.02–0.44	0.02–0.69	0–0.02
		F	Adult (>10 yrs)	48–49 ^{b,c}	7.28–14.47	2.99–8.04	1.96–7.27	0.06–0.40	0.07–0.63	0–0.01

^aCommercial laboratory.^bCoulter Counter S770 and Serono Baker System 9000S.^cInterval represents 5th–95th percentile.

TABLE 110.18 Selected Erythrocyte and Leukocyte Parameters for Baboons (Mean ± SD and/or Range)

Reference	Year	Species	Sex	Age	No.	RBC ($\times 10^6/\mu\text{L}$)	Hgb (g/dL)	Hct (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	Platelets ($\times 10^3/\mu\text{L}$)	MPV (fL)	RDW (%)	Reticulocytes (%)		
49	2004	<i>Papio</i> sp.	M	All	41 ^a	5.13 ± 0.25	12.9 ± 0.55	40.0 ± 1.7	77.9 ± 2.6	25.17 ± 0.99	32.28 ± 0.85	390 ± 70	—	—	—		
			F	All	44 ^a	5.05 ± 0.37	13.06 ± 0.81	40.3 ± 2.5	79.9 ± 2.2	25.87 ± 0.75	32.39 ± 0.75	346 ± 113	—	—	—		
18	2003	<i>Papio hamadryas</i>	M/F	Infant	50–110 ^b	5.24 ± 0.52 (3.63–6.29)	13.21 ± 1.3 (9.8–18.0)	40.8 ± 3.9 (30.9–55.0)	78.5 ± 7.1 (61.8–97.1)	25.4 ± 2.6 (18.8–32.1)	32.4 ± 0.7 (30.4–34.2)	411 ± 133 (165–732)	8.7 ± 1.0 (7.1–12.0)	13.5 ± 1.5 (11.0–18.4)	—		
15	1982	<i>Papio</i> sp.	M	Adult (6–14 yrs)	16	5.06 ± 0.38 4.46–5.76	13.4 ± 1.0 (12.1–15.3)	41 ± 4 (38–48)	82 ± 4 (75–92)	26 ± 1 (25–28)	33 ± 2 (30–36)	334 ± 73 (205–451)	—	—	1.1 ± 0.3 (0.6–1.5)		
			F	Adult (6–15 yrs)	15–16	4.55 ± 0.38 (3.84–5.43)	11.7 ± 0.7 (10.4–12.9)	38 ± 2 (35–42)	83 ± 4 (77–93)	26 ± 2 (24–29)	31 ± 1 (29–34)	333 ± 136 140–597	—	—	1.1 ± 0.4 (0.3–1.7)		
			M/F	Juv (1–5 yrs)	43	4.97 ± 0.28 (4.25–5.69)	12.6 ± 0.9 (8.7–13.9)	40 ± 2 (31–43)	80 ± 5 (63–90)	26 ± 2 (18–27)	32 ± 1 (28–34)	399 ± 76 (225–544)	—	—	1.2 ± 0.4 (0.3–2.3)		
Reference	Year	Species	Sex	Age	No.	WBC ($\times 10^3/\mu\text{L}$)	Neutrophils ($\times 10^3/\mu\text{L}$)	Lymphocytes ($\times 10^3/\mu\text{L}$)	Monocytes ($\times 10^3/\mu\text{L}$)	Eosinophils ($\times 10^3/\mu\text{L}$)	Basophils ($\times 10^3/\mu\text{L}$)	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)	Basophils (%)	
49	2004	<i>Papio</i> sp.	M	All	41	7.6 ± 2.3	2.8 ± 1.4	4.4 ± 1.4	0.25 ± 0.22	0.06 ± 0.07	0.02 ± 0.04	37 ± 11	59 ± 10	—	—	—	
			F	All	44	8.2 ± 2.5	3.8 ± 2.2	4.1 ± 1.2	0.29 ± 0.21	0.08 ± 0.13	0.01 ± 0.04	43 ± 15	52 ± 15	—	—	—	
18	2003	<i>Papio hamadryas</i>	M/F	Infant	88–110	11.63 ± 4.47 (2.90–25.30)	—	—	—	—	—	52.3 ± 17.6 (21.5–90.9)	45.0 ± 16.7 (6.7–78.0)	3.3 ± 2.4 (0.2–10)	1.0 ± 0.7 (0–4.2)	0.5 ± 0.6 (0–3.1)	
15	1982	<i>Papio</i> sp.	M	Adult (6–14 yrs)	16	10.9 ± 4.0 (5.9–20.8)	—	—	—	—	—	54 ± 20 (22–85)	42 ± 20 (12–75)	1 ± 1.2 (0–4)	2 ± 1.7 (0–5)	0.1 ± 0.3 (0–1)	
			F	Adult (6–15 yrs)	15–16	10.8 ± 3.0 (6.4–17.1)	—	—	—	—	—	—	64 ± 21 (22–87)	33 ± 19 (13–77)	1 ± 1.1 (0–3)	1 ± 0.9 (0–2)	0.1 ± 0.3 (0–1)
			M/F	Juv (1–5 yrs)	43	7.6 ± 2.7 (3.3–19.0)	—	—	—	—	—	—	44 ± 13 (23–78)	53 ± 13 (14–76)	0.5 ± 0.7 (0–3)	1.6 ± 1.9 (0–8)	0.1 ± 0.2 (0–1)

^aCell-Dyn 3500.

^bCoulter Counter, JT or MAXM.

TABLE 110.19 Selected Erythrocyte and Leukocyte Parameters for Cynomolgus Monkeys (Mean ± SD)

Reference	Year	Strain	Sex	Age	No.	RBC ($\times 10^6/\mu\text{L}$)	Hgb (g/dL)	Hct (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	Platelets ($\times 10^3/\mu\text{L}$)	MPV (fL)	Reticulocytes (%)
33	2008	Chinese	F	2.5–6.5 yrs	1445 ^a	5.17 ± 0.41	12.3 ± 0.9	39.1 ± 3.0	75.8 ± 3.6	23.8 ± 1.3	31.4 ± 1.1	362 ± 90	9.0 ± 1.4	—
			M	2.5–6.5 yrs	1445 ^a	5.39 ± 0.41	12.6 ± 0.9	40.2 ± 3.0	74.7 ± 3.4	23.5 ± 1.3	31.5 ± 1.3	360 ± 92	8.9 ± 1.5	—
48	2005	Not given	F	N/A	53 ^b	6.22 ± 0.71	11.9 ± 0.7	38.8 ± 3.7	62.7 ± 3.7	19.4 ± 3.1	30.8 ± 3.1	400 ± 90	—	0.6 ± 0.3
			M	N/A	53 ^b	6.78 ± 0.56	12.6 ± 0.8	41.9 ± 3.0	61.9 ± 3.8	18.6 ± 1.2	30.1 ± 1.2	460 ± 120	—	0.4 ± 0.3
55	1986	Not given	M/F	1 day	5 ^c	6.04 ± 0.64	15.0 ± 1.6	52.6 ± 6.6	88.0 ± 6.1	—	—	—	—	—
			M/F	1 mo	13 ^c	5.53 ± 0.57	12.3 ± 1.3	41.2 ± 2.8	74.5 ± 5.8	—	—	—	—	—
			M/F	5 mo	11 ^c	6.95 ± 0.84	11.8 ± 1.1	47.5 ± 4.3	68.0 ± 4.1	—	—	—	—	—
			M/F	8 mo	11 ^c	6.96 ± 0.44	12.0 ± 1.4	47.3 ± 3.0	68.1 ± 2.7	—	—	—	—	—
			M/F	10 mo	9 ^c	6.69 ± 0.96	13.5 ± 3.3	45.7 ± 4.4	68.2 ± 4.5	—	—	—	—	—

Reference	Year	Strain	Sex	Age	No.	WBC ($\times 10^3/\mu\text{L}$)	Neutrophils ($\times 10^3/\mu\text{L}$)	Lymphocytes ($\times 10^3/\mu\text{L}$)	Monocytes ($\times 10^3/\mu\text{L}$)	Eosinophils ($\times 10^3/\mu\text{L}$)	Basophils ($\times 10^3/\mu\text{L}$)	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)	Basophils (%)
33	2008	Chinese	F	2.5–6.5 yrs	1445 ^a	11.2 ± 4.8	—	—	—	—	—	—	—	—	—	—
			M	2.5–6.5 yrs	1445 ^a	11.5 ± 4.0	—	—	—	—	—	—	—	—	—	—
48	2005	Not given	F	N/A	53 ^b	11.2 ± 3.9	4.5 ± 1.9	5.6 ± 2.2	0.7 ± 0.3	0.3 ± 0.3	0.03 ± 0.04	40.0 ± 8.0	51.0 ± 7.0	—	—	—
			M	N/A	53 ^b	12.6 ± 3.8	5.6 ± 2.7	5.6 ± 2.0	0.9 ± 0.4	0.4 ± 0.3	0.04 ± 0.05	44.0 ± 12.0	45.0 ± 12.0	—	—	—
55	1986	Not given	M/F	1 day	5 ^c	9.2 ± 2.7	—	—	—	—	—	56.0 ± 10.3	38.8 ± 10.0	4.1 ± 2.4	0.8 ± 0.6	0.1 ± 0.3
			M/F	1 mo	13 ^c	9.5 ± 3.0	—	—	—	—	—	18.9 ± 11.3	75.3 ± 12.2	3.8 ± 3.3	1.3 ± 1.6	0.4 ± 0.7
			M/F	5 mo	11 ^c	12.8 ± 3.2	—	—	—	—	—	33.0 ± 6.6	64.6 ± 5.9	1.0 ± 1.5	1.0 ± 0.9	0.2 ± 0.4
			M/F	8 mo	11 ^c	13.4 ± 2.6	—	—	—	—	—	26.5 ± 10.4	70.4 ± 10.8	1.7 ± 2.1	1.4 ± 1.6	0.1 ± 0.3
			M/F	10 mo	9 ^c	13.5 ± 3.3	—	—	—	—	—	16.7 ± 3.1	78.3 ± 3.5	2.4 ± 1.2	2.4 ± 0.1	0.1 ± 0.3

^aAdvia 120.

^bCell-Dyn 3500.

^cToa Medical Electronics Co Ltd., Type CC-108, 110 and Beckman Coulter S-Plus IV.

TABLE 110.20 Selected Erythrocyte and Leukocyte Parameters for Rhesus Monkeys (Range and/or Mean \pm SD)

Reference	Year	Sex	Age	No.	RBC ($\times 10^6/\mu\text{L}$)	Hgb (g/dL)	Hct (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	RDW (%)	Platelets ($\times 10^3/\mu\text{L}$)	MPV	Reticulocytes ($\times 10^6/\text{mL}$)
51	2001	M/F	7–36 yrs	771–3322 ^a	5.62 \pm 0.69	13.4 \pm 1.6	41.6 \pm 4.7	74.6 \pm 4.9	24.1 \pm 1.9	32.4 \pm 4.8	—	—	—	—
5	1997	F	<1 yr	27 ^b	6.04 \pm 0.46	12.8 \pm 0.8	40.4 \pm 2.5	67.2 \pm 3.9	21.3 \pm 1.4	31.6 \pm 0.7	—	—	—	—
		M	<1 yr	27 ^b	6.18 \pm 0.56	13.1 \pm 0.8	41.4 \pm 3.2	67.1 \pm 3.0	21.3 \pm 1.1	31.7 \pm 0.8	—	—	—	—
		F	1–2 yrs	77 ^b	5.75 \pm 0.36	12.9 \pm 0.7	40.1 \pm 2.2	69.4 \pm 4.2	22.4 \pm 0.9	32.0 \pm 1.2	—	—	—	—
		M	1–2 yrs	30 ^b	5.73 \pm 0.39	12.8 \pm 0.7	40.0 \pm 2.2	69.8 \pm 2.6	22.4 \pm 1.0	32.0 \pm 0.6	—	—	—	—
		F	2–3 yrs	50 ^b	5.49 \pm 0.41	12.6 \pm 0.8	39.0 \pm 2.5	71.1 \pm 2.6	23.0 \pm 1.1	32.3 \pm 0.6	—	—	—	—
		M	2–3 yrs	27 ^b	5.71 \pm 0.31	13.0 \pm 0.6	39.7 \pm 2.0	68.4 \pm 6.1	22.7 \pm 0.9	32.7 \pm 0.7	—	—	—	—
		F	3–4 yrs	25 ^b	5.56 \pm 0.46	12.5 \pm 0.7	38.6 \pm 2.3	69.6 \pm 2.9	22.6 \pm 1.0	32.4 \pm 0.7	—	—	—	—
		M	3–4 yrs	30 ^b	5.79 \pm 0.42	12.9 \pm 0.7	40.2 \pm 2.8	69.5 \pm 2.4	22.4 \pm 1.1	31.9 \pm 1.8	—	—	—	—
		F	4–5 yrs	13 ^b	5.85 \pm 0.42	12.8 \pm 0.7	39.5 \pm 2.5	67.6 \pm 2.5	22.0 \pm 0.8	32.6 \pm 0.5	—	—	—	—
		M	4–5 yrs	44 ^b	5.89 \pm 0.35	13.2 \pm 0.8	41.1 \pm 2.8	69.7 \pm 2.8	22.4 \pm 0.9	32.2 \pm 0.6	—	—	—	—
		F	5–10 yrs	30 ^b	5.75 \pm 0.41	12.9 \pm 0.8	40.3 \pm 2.6	70.4 \pm 3.6	22.4 \pm 1.3	31.9 \pm 1.8	—	—	—	—
		M	5–10 yrs	21 ^b	6.90 \pm 0.34	13.6 \pm 0.7	42.4 \pm 2.5	70.7 \pm 2.2	22.8 \pm 0.9	32.2 \pm 0.8	—	—	—	—
F	>10 yrs	29 ^b	6.01 \pm 0.72	13.6 \pm 0.9	42.1 \pm 2.6	70.0 \pm 2.6	23.7 \pm 1.1	32.4 \pm 1.0	—	—	—	—		
12	1994	F	Adult	15 ^{c,d}	4.96–6.38	12.1–14.9	37–46	69.0–77.0	22.1–25.3	31.2–33.8	12.4–15.5	314–699	8.0–10.9	61.1–190.4
		M	Adult	20 ^{c,d}	5.12–7.00	12.2–16.0	38–48	68.7–79.7	22.0–25.9	31.0–33.5	12.7–15.4	183–580	7.9–11.4	39.6–175.5
		M/F	Infant	9 ^{c,d}	4.55–5.91	11.0–13.7	34–42	69.6–76.3	22.8–24.8	31.5–33.6	12.7–15.5	341–672	7.6–11.5	27.4–167.3
29	1983	M	6–14 yrs	10 ^a	6.0 \pm 0.3	14.1 \pm 0.7	47.8 \pm 1.6	79.9 \pm 2.1	23.4 \pm 0.9	29.4 \pm 1.0	—	—	—	—
		M	15–28 yrs	7 ^a	6.3 \pm 1.2	14.3 \pm 2.8	46.7 \pm 8.1	74.7 \pm 2.9	22.6 \pm 1.0	30.4 \pm 0.8	—	—	—	—
		F	4–10 yrs	24 ^a	5.8 \pm 0.6	13.4 \pm 1.4	45.4 \pm 3.7	79.9 \pm 4.0	23.4 \pm 1.5	29.2 \pm 1.0	—	—	—	—
		F	16–25 yrs	17 ^a	5.9 \pm 0.7	12.5 \pm 1.4	42.0 \pm 4.6	72.0 \pm 4.4	21.5 \pm 1.9	29.8 \pm 1.9	—	—	—	—

TABLE 110.20 Selected Erythrocyte and Leukocyte Parameters for Rhesus Monkeys (Range and/or Mean ± SD) (Continued)

Reference	Year	Sex	Age	No.	WBC ($\times 10^3/\mu\text{L}$)	Neutrophils ($\times 10^3/\mu\text{L}$)	Lymphocytes ($\times 10^3/\mu\text{L}$)	Monocytes ($\times 10^3/\mu\text{L}$)	Eosinophils ($\times 10^3/\mu\text{L}$)	Basophils ($\times 10^3/\mu\text{L}$)	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)
51	2001	M/F	7–36 yrs	771–3322 ^a	7.84 ± 3.50	3.67 ± 2.15	2.43 ± 1.09	0.21 ± 0.16	0.21 ± 0.17	—	51.5 ± 15.9	38.9 ± 14.6	3.5 ± 2.3	3.9 ± 3.6
5	1997	F	<1 yr	27 ^b	11.2 ± 4.3	6.00 ± 3.17	4.75 ± 2.67	0.26 ± 0.24	0.07 ± 0.10	0.04 ± 0.08	—	—	—	—
		M	<1 yr	27 ^b	9.5 ± 3.0	4.79 ± 2.49	4.37 ± 1.91	0.21 ± 0.17	0.03 ± 0.05	0.02 ± 0.04	—	—	—	—
		F	1–2 yrs	77 ^b	9.8 ± 3.4	5.96 ± 3.03	3.44 ± 1.73	0.28 ± 0.27	0.04 ± 0.08	0.004 ± 0.02	—	—	—	—
		M	1–2 yrs	30 ^b	9.8 ± 3.5	6.37 ± 2.86	2.85 ± 1.61	0.25 ± 0.25	0.03 ± 0.05	0.005 ± 0.02	—	—	—	—
		F	2–3 yrs	50 ^b	9.7 ± 3.3	5.64 ± 2.89	3.40 ± 1.71	0.32 ± 0.26	0.08 ± 0.12	0.02 ± 0.04	—	—	—	—
		M	2–3 yrs	27 ^b	8.9 ± 2.1	5.12 ± 1.71	3.53 ± 1.69	0.16 ± 0.15	0.03 ± 0.05	0.006 ± 0.02	—	—	—	—
		F	3–4 yrs	25 ^b	10.6 ± 3.3	6.60 ± 2.95	3.63 ± 1.54	0.19 ± 0.19	0.09 ± 0.15	0.01 ± 0.06	—	—	—	—
		M	3–4 yrs	30 ^b	10.5 ± 2.8	6.03 ± 2.32	3.91 ± 1.24	0.37 ± 0.39	0.12 ± 0.22	0.005 ± 0.02	—	—	—	—
		F	4–5 yrs	13 ^b	11.6 ± 3.1	7.68 ± 2.96	3.59 ± 1.10	0.19 ± 0.21	0.08 ± 0.11	0 ± 0	—	—	—	—
		M	4–5 yrs	44 ^b	10.4 ± 2.6	6.37 ± 2.32	3.59 ± 1.59	0.29 ± 0.25	0.13 ± 0.20	0.007 ± 0.03	—	—	—	—
		F	5–10 yrs	30 ^b	10.3 ± 3.3	6.92 ± 3.16	3.65 ± 4.66	0.36 ± 0.26	0.12 ± 0.20	0.01 ± 0.06	—	—	—	—
12	1994	F	Adult	15 ^{c,d}	4.4–11.1	0.90–7.05	1.68–5.76	0.07–0.94	0–0.99	0–0.13	—	—	—	—
		M	Adult	20 ^{c,d}	2.6–10.0	0.97–6.50	1.27–5.93	0.08–0.78	0.02–0.73	0–0.10	—	—	—	—
		M/F	Infant	9 ^{c,d}	3.0–9.8	0.79–7.59	1.33–5.84	0.05–0.61	0–0.18	0–0.1	—	—	—	—
29	1983	M	6–14 yrs	10 ^a	12.1 ± 6.5	—	—	—	—	—	67.2 ± 8.2	29.1 ± 10.0	2.3 ± 1.3	0.9 ± 1.7
		M	15–28 yrs	7 ^a	12.1 ± 3.7	—	—	—	—	—	67.3 ± 12.3	23.3 ± 10.7	1.7 ± 1.0	7.3 ± 6.3
		F	4–10 yrs	24 ^a	19.8 ± 5.2	—	—	—	—	—	81.0 ± 4.8	14.7 ± 4.8	2.0 ± 1.5	1.6 ± 1.6
		F	16–25 yrs	17 ^a	14.1 ± 7.6	—	—	—	—	—	65.1 ± 17.4	26.5 ± 15.2	2.5 ± 1.8	5.5 ± 5.0

^aCommercial laboratories.

^bBaker System 9000 Hematology Analyzer.

^cCoulter Counter model S and S-Plus IV.

^dRange expressed as difference between 2.5 and 97.5 percentiles.

TABLE 110.21 Coagulation Reference Intervals for Selected Species

Reference	Year	Species	Age	No.	Statistic	PT (sec)	APTT (sec)	Fibrinogen (mg/dL)	RVVT (sec)	Clotting Time (min)	D-dimer (µg/mL)	TAT Complex (µg/L)
40	2007	<i>Macaca fascicularis</i> (Mauritian)	2 yrs M/F	8	Range	13.1–13.9	25.7–34.2	—	—	—	0.40–1.68	3.5–14.6
11	2006	<i>Papio anubis</i>	5 M	9	Mean ± SD	—	—	149.0 ± 25.6	—	—	1.25 ± 0.71	16.4 ± 10.2
16	1993	<i>Papio sp.</i>	4–26 yrs M	12	2 SD Range	11.2–15.0	28.6–39.4	134–222	—	—	—	—
		<i>Papio sp.</i>	4–26 yrs F	20	2 SD Range	11.9–13.9	26.9–35.3	112–204	—	—	—	—
		<i>Pan troglodytes</i>	5–33 yrs M	20	2 SD Range	10.2–13.2	17.7–24.5	223–505	—	—	—	—
		<i>Pan troglodytes</i>	5–33 yrs F	30	2 SD Range	10.9–12.7	17.7–24.1	225–485	—	—	—	—
32	1996	<i>Pan troglodytes</i>	Adult M/F	5	Mean ± SD	10.6 ± 0.4	22.0 ± 1.8	—	15.9 ± 1.4	3 ± 1.0 ^c	—	—
		<i>Papio sp.</i>	Adult M	6–9	Mean ± SD	12.1 ± 0.5	33.1 ± 4.1	—	17.6 ± 1.7	6 ± 0.9 ^c	—	—
		<i>Macaca mulatta</i>	Adult M/F	10	Mean ± SD	11.5 ± 1.4	35.5 ± 2.9	—	21.6 ± 1.1	5 ± 1.3 ^c	—	—
36	1985	<i>Callithrix jacchus</i>	Adult M	13	Mean ± SEM	8.3 ± 0.5	29.7 ± 1.4	162 ± 17	15.9 ± 1.2	2.08 ± 0.8	—	—
			Adult F	13	Mean ± SEM	8.0 ± 0.5	32.3 ± 1.1	235 ± 19	19.0 ± 1.6	2.31 ± 0.12	—	—
38	1984	<i>Aotus trivirgatus</i>	All M/F	28	Mean ± SD	—	25.9 ± 3.1 ^a	—	—	—	—	—
					Mean ± SD	—	19.6 ± 1.8 ^b	—	—	—	—	—
20	1983	<i>Saguinus oedipus</i>	Adult M/F	24	Range	—	—	250–520	—	—	—	—
19	1982	<i>Callithrix jacchus</i>	All M/F	27	2SD Range	—	—	215–399	—	—	—	—

^aUsing diatomaceous earth.

^bUsing ellagic acid activator.

^cGlass.

TABLE 110.22 Summary of Reported Bone Marrow Data for Selected Species (Mean)

Reference	Year	Sex	Age	Species	No.	Granulocytic Series (%)	Erythroid Series (%)	Lymphocytic Series (%)	Plasmacytic Series (%)	M:E Ratio
43	1998	M	Adult	Cyno	11	47.60	46.05	5.44	1.45	1.03
	1998	F	Adult	Cyno	12	47.28	46.28	5.12	1.49	1.02
44	1993	M	3–4 yrs	Rhesus	3	46.20	46.80	5.70	1.40	1.00
	1993	F	3–4 yrs	Rhesus	3	40.50	51.30	6.30	1.40	0.80
3	1973	M/F	Adult	Baboon	N/A	55.9	31.2	13.1	0.20	1.79
56	1967	M/F	Adult	Rhesus	25	53.04	39.12	4.49	2.71	1.36
	1967	M	Adult	Rhesus	5	52.54	40.74	4.20	1.82	1.29
	1967	F	Adult	Rhesus	20	53.17	38.72	4.56	2.93	1.37
41	1962	N/A	N/A	Rhesus	14	48.90	24.80	24.60	1.14	1.97

Although many prosimian NHPs are nocturnal, owl monkeys are the only nocturnal species of simian NHPs and the only nocturnal NHP species that is commonly used in research. Diurnal variation in both RBC and WBC parameters is due to circadian rhythms and alterations in hydration. These factors should be taken into consideration when sampling blood from owl monkeys.

Leukocytes

A recent report calculated the average diameter of WBCs from a group of macaques that consisted of four species (rhesus, cynomolgus, stump-tailed, and pig-tailed). Neutrophils and eosinophils measured 10.7–13.3 μm , lymphocytes measured 9.5–12.5 μm , monocytes measured 12.5–15.5 μm and basophils measured 10.5–13.0 μm .⁴⁵

Neutrophils

Neutrophils generally exceed lymphocytes in the peripheral blood of normal adult chimpanzees, baboons, rhesus monkeys, and African green monkeys (Tables 110.16–110.20). The typical NHP neutrophil contains a condensed, twisted, or segmented nucleus that commonly presents 5–6 lobes but may possess as many as 10–12 (Fig. 110.12A, D, E, H). A small drumstick-shaped appendage (Barr body) is attached to the nucleus of neutrophils from females. The cytoplasm varies from pale to light pink, and granulation is a feature of most, if not all, neutrophils from NHPs. The color of eosinophil granules has been reported to vary from eosinophilic to azurophilic to basophilic in cotton-top tamarins, squirrel monkeys, and chimpanzees respectively.^{6,7,19}

Lymphocytes

Lymphocytes exceed neutrophils in the peripheral blood of normal adult cynomolgus monkeys and in many New World monkeys including owl monkeys, squirrel monkeys, marmosets, and some species of tam-

arins (Tables 110.11–110.15, 110.19). Lymphocytes from most NHP species resemble human lymphocytes, although “atypical” or “reactive cells” may be seen (Fig 110.12A, B, D).

Monocytes

Monocytes in NHPs are larger than the lymphocytes and may appear in two forms. The first type of monocyte has a broader band of cytoplasm than is observed in the lymphocytes, with a mottled gray-blue color with a foamy appearance or with discrete vacuoles (Fig. 110.12A). The second type of monocyte, a transitional form, has a large oval nucleus devoid of depressions, and its cytoplasm appears darker adjacent to the nucleus and often has delicate pale reddish granulation. In general, monocyte counts are low in healthy NHPs, typically ranging from 0% to 4% of the total WBC count.

Eosinophils

As compared to other mammalian species, eosinophils and neutrophils are somewhat harder to distinguish due to their equivalent size and the presence of azurophilic or eosinophilic granules in both cell types. Eosinophils can be confidently identified using the following criteria: (1) eosinophil granules are almost twice as large and rounder than those of neutrophils; (2) eosinophil granules have a brighter yellow-red color; and may be superimposed on the nucleus; (3) the cytoplasm of eosinophils is slightly more gray-blue; and (4) the eosinophil nucleus is not as condensed or segmented (Fig. 110.12E, F, H).⁴⁷ Marmoset eosinophils contain irregular spherical granules which often have a bluish tinge and create a “muddy” gray-blue cytoplasm that can be confused with basophils.

Idiopathic eosinophilia has been described for specific owl monkey karyotypes. Early studies excluded parasitism and subclinical disease as a cause of the eosinophilia and suggested a genetic component. A more recent study analyzed eosinophils from two karyo-

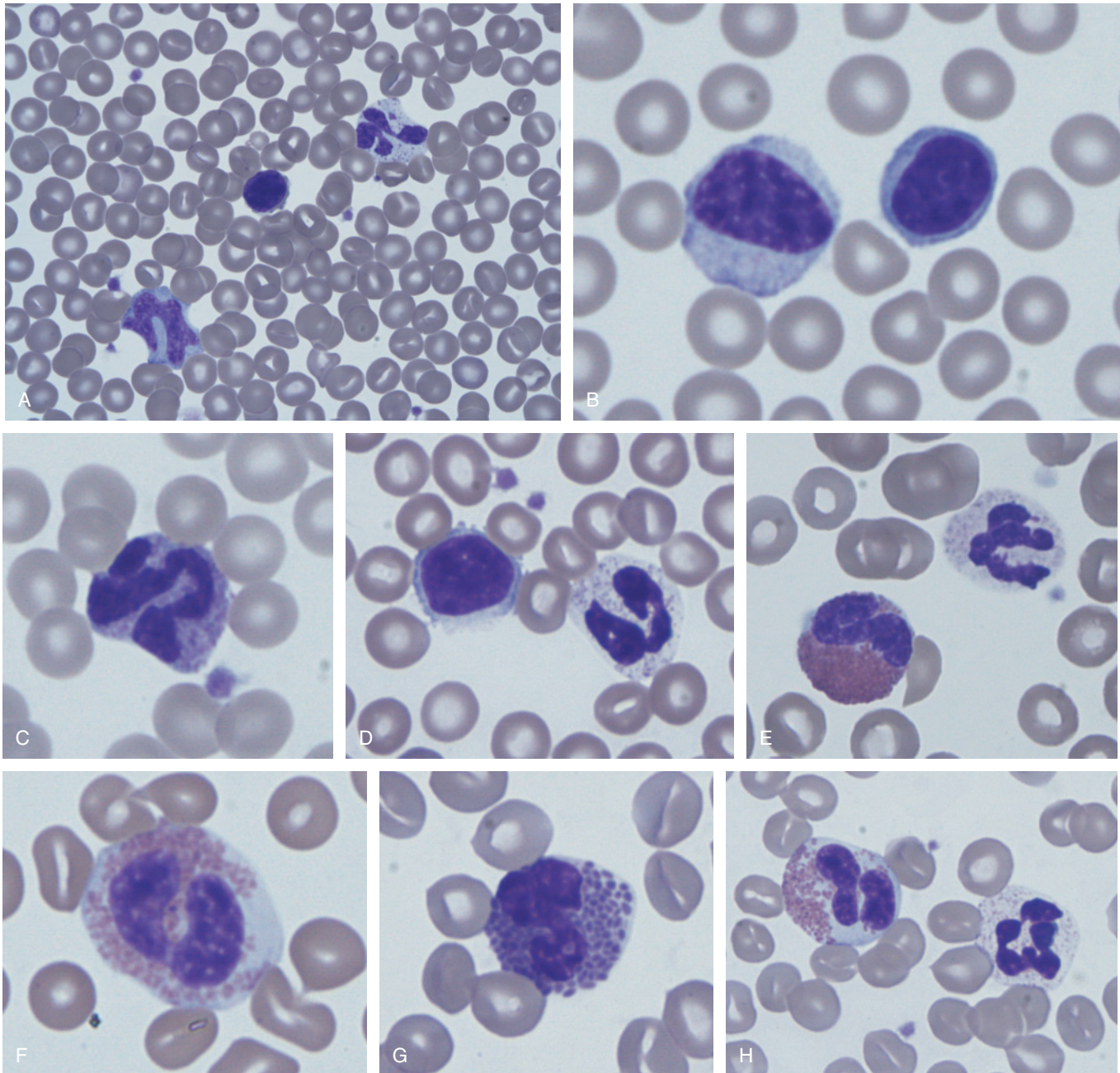


FIGURE 110.12 Peripheral blood cells: (A) Owl monkey (*Aotus nancymae*) – from left to right a monocyte, lymphocyte and neutrophil. (B) Bolivian squirrel monkey (*Saimiri boliviensis*) – two lymphocytes. (C) Bolivian squirrel monkey (*Saimiri boliviensis*) – basophil. (D) Bolivian squirrel monkey (*Saimiri boliviensis*) – lymphocyte and neutrophil. (E) Chimpanzee (*Pan troglodytes*) – eosinophil and neutrophil. (F) Guyanese squirrel monkey (*Saimiri sciureus sciureus*) – eosinophil. (G) Rhesus macaque (*Macaca mulatta*) – basophil. (H) Rhesus macaque (*Macaca mulatta*) – eosinophil and neutrophil. Modified Wright-Giemsa stain.

types, K-I (*Aotus nancymae*) and K-VI (*Aotus azarae boliviensis*) that are associated with low and high levels of eosinophils, respectively. The results indicated that the genetic basis of the eosinophilia in K-VI monkeys was likely a function of heightened eosinophilopoiesis and depressed recruitment kinetics from the peripheral circulatory pool in response to RANTES.¹

Basophils

Basophils contain dull gray cytoplasm with dull gray rods and additional pale violet to dark purple, round or rod-like granules of varying sizes (Fig 110.12C, G). The granules typically do not obscure the nucleus, but the morphology and number of the granules may vary

significantly between species. As in many other mammalian species, basophils are present in low numbers in the blood and typically account for 0–2% of the total WBC count.

Platelets

The morphology of platelets from NHP species is similar to platelets from other species. They display an amorphous pale-blue ground substance and pronounced violet granulation. Transmission electron micrographs of chimpanzee and monkey platelets show that most of the internal structures are similar to those seen in many mammalian species with the exception of large clumps of glycogen and structures of unknown character circumscribed with fibrous strands.³² Platelet numbers vary between species. Macaques frequently have higher platelet counts than humans, while chimpanzee platelet counts tend to be lower than humans and other primate species.¹² Although few reports on platelet activity in NHPs exist, differences between the activity of human platelets have been cited. Platelets from baboons and chimpanzees reportedly aggregate with collagen, ristocetin, and ADP. Biphasic aggregation (i.e. aggregation followed quickly by disaggregation) to ADP is common in both species; however, chimpanzee platelets are not responsive to arachidonic acid.³² Platelets from rhesus monkeys aggregate in response to collagen, ADP and arachidonic acid in a manner similar to humans.^{25,57} Marmoset platelets aggregate with ristocetin and collagen at higher concentrations than human platelets but fail to respond to epinephrine. In addition, they aggregate with arachidonate at concentrations similar to human platelets and have a monophasic response to ADP.³⁶

Coagulation

Reported coagulation values from healthy animals of multiple NHP species are provided in Table 110.21. In general, coagulation values for most species are similar to those reported for humans. Comparisons between the clotting system of rhesus macaques, cynomolgus macaques, and humans concluded that both NHP species are suitable for studying coagulation defects that occur in humans. Specific differences included higher levels of factors II, VII, VIII, and XII in rhesus macaques and lower levels of factor IX activity in both rhesus and cynomolgus macaques as compared to humans.^{4,50} Activation of fibrinolysis, intrinsic coagulation, prothrombin consumption, and functional levels of most coagulation factors in cynomolgus macaques were very similar to humans.⁴ Clotting times for baboons, chimpanzees, and macaques are typically short, with clots being retracted partially or completely by 4 hours.³² A recent comparison of 18 analytes that assessed excessive thrombin activation, clotting inhibition, endothelial damage, and fibrinolysis between normal baboons and humans showed a high level of concordance. Exceptions included elevated thrombin/

antithrombin complex and fibrinopeptide A and lower plasminogen activity in NHPs.¹¹

Aging

Studies designed to determine the effects of aging on hematologic indices have generated conflicting results. Studies by Smucny et al.⁵¹ and Nakamura et al.³⁹ concluded that WBC counts are unchanged in aged rhesus monkeys up to 30 years old, while a third study by Kessler et al.²⁹ identified decreased WBC counts in aged male and female rhesus monkeys (15–28 years). In addition, Smucny et al.⁵¹ and Nakamura et al.³⁹ noted general increases in Hct and Hgb for monkeys over 20 years old, while Kessler et al.²⁹ documented lower Hcts and MCH for aged female rhesus monkeys (15–28 years). All studies concurred that MCV generally declined with age.

Bone Marrow Evaluation and Cytology

In larger NHPs, bone marrow can be collected from a number of sites including the iliac crest, the ischial tuberosity, the trochanteric fossa of the femur, the tibial crest or the proximal humerus.¹³ The trochanteric fossa and the proximal humerus are useful for collection from smaller animals. The site of bone marrow aspiration and the technique used may affect the percentage of hematopoietic cells. In cynomolgus monkeys, samples collected by conventional aspiration from the ilium contained greater than 20% total T cells, while perfusion aspirates from the long bones (humerus and femur) contained less than 4% T cells.³¹ This difference is probably due to blood contamination.

Bone marrow constituents are reportedly similar when sampled from three separate sites: sternum, ribs, and femur.⁴⁷ With the exception of rhesus macaques, the literature contains few reports on the normal cellular composition of NHP bone marrow. Table 110.22 details the published results for quantification of bone marrow populations in the rhesus macaque, cynomolgus macaque, and baboon. For these species the M:E ratio was notably lower than humans, in which the reference interval is approximately 2.0–4.0. Some reported values for rhesus macaques were variable. The M:E ratio ranged from 0.8 to 1.97.^{41,44} Most authors reported a lymphocyte count between 4% and 9% while one author reported 24.6% lymphocytes.⁴¹ This discrepancy may have resulted from blood contamination.

The morphology of the hematopoietic cell populations has been described for cynomolgus monkeys.⁴³ Several morphologic descriptions of hematopoietic cells from rhesus monkeys indicate strong similarity between the two species.⁵⁶ Examples of different hematopoietic cell types from the rhesus monkey are provided in Fig. 110.13. A unique feature of bone marrow from rhesus and cynomolgus macaques is the presence of “giant” band neutrophils and metamyelocytes.^{43,56} Marrow from both species contains stainable iron, rare monocytes, monocyte precursors, and less than 1% non-hematopoietic elements. Lymphocyte, plasma cell,

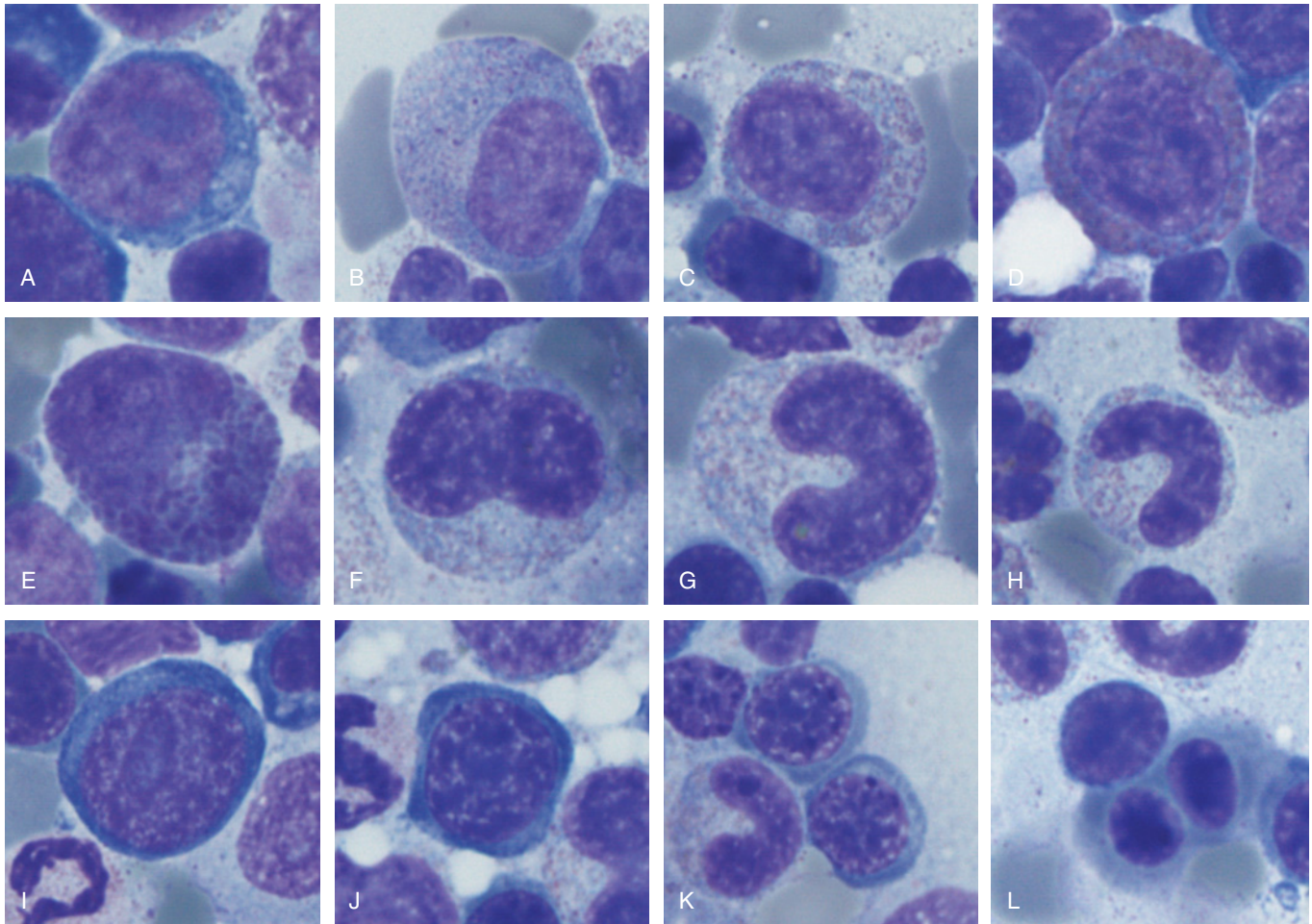


FIGURE 110.13 Rhesus monkey bone marrow aspirate: (A) myeloblast; (B) promyelocyte; (C) neutrophilic myelocyte; (D) eosinophilic myelocyte; (E) basophilic myelocyte; (F) metamyelocyte; (G) giant band neutrophil; (H) band neutrophil; (I) rubriblast; (J) prorubricyte; (K) two rubricytes; (L) two metarubricytes and one lymphocyte. Modified Wright-Giemsa stain.

monocyte, and megakaryocyte morphology is similar to humans and other mammalian species.

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Hematology of the Ferret

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Blood Collection

Morphology and Numbers of Peripheral Blood Cells

Erythrocytes

Platelets

Leukocytes

Bone Marrow Cytology

Other Hematologic Values

Acronyms and Abbreviations

Hgb, hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean cell volume; M:E, myeloid to erythroid ratio; PCV, packed cell volume; RBCs, red blood cells; WBC, white blood cell count.

BLOOD COLLECTION

Several reviews have described a number of methods for collection of blood from ferrets, including toenail clipping (yielding less than 0.5 mL of blood), retro-orbital sinus blood collection (safely yielding 1–3 mL of blood in ferrets weighing 100–300 g and 5–10 mL of blood from adults), cardiac puncture, caudal arterial puncture in the tail, and jugular venipuncture.^{2–4,6,9,11,18,19,27,29,30,34} Chronic jugular catheterization for repeated blood sampling has also been described.^{10,26}

The total blood volume of the ferret is estimated to be between 5 and 7% of body mass, resulting in a total blood volume in adult males of approximately 60 mL and approximately 40 mL in adult females.^{13,15,24} Terminal blood collection (exsanguination) from the abdominal aorta in anesthetized ferrets will yield approximately 3% of body mass in blood.³⁵

MORPHOLOGY AND NUMBERS OF PERIPHERAL BLOOD CELLS

Reported hematologic reference intervals for the ferret (*Mustela putorius furo*) are provided in Tables 111.1 and 111.2. Additional reported values for ferrets can be found in the literature.^{7,8,12,17,21,28,37} As a cautionary note, the hematocrit, white blood cell (WBC) numbers, and platelet findings from anesthetized ferrets can be significantly less than reported reference intervals.^{23,25}

Erythrocytes

The mean diameters reported for red blood cells (RBCs) of ferrets were found to be 5.94 μm in males (range, 4.6–7.7 μm) and 6.32 μm in females (range, 4.6–7.7 μm).^{14,24,34,35} Hematocrit and RBC count values for ferrets were found to be higher than indices for dogs and cats.¹³ Thornton et al. observed the range of reticulocytes in albino ferrets to be 1–12% (mean, 4%) in males and 2–14% (mean, 5.3%) in females.³⁵ Howell-Jolly bodies were seen in about 5% of male and female ferrets but were not observed in large numbers in those animals^{34,35} (Fig 111.1).

Platelets

The mean diameters reported for platelets of ferrets were found to be 1.7 μm (range, 1.5–2.3 μm) in males and females (Fig. 111.1).^{14,35} Kawasaki provided reference intervals of circulating platelets from ferrets seen in his practice, 297–730 $\times 10^3/\mu\text{L}$ (mean, 453 $\times 10^3/\mu\text{L}$), and a range suggested by his colleagues, 350–550 $\times 10^3/\mu\text{L}$ (mean, 400 $\times 10^3/\mu\text{L}$).²⁰ Thornton et al. observed the following platelet numbers in adult albino ferrets: 297–730 $\times 10^3/\mu\text{L}$ (mean, 453 $\times 10^3/\mu\text{L}$) in males, and 310–910 $\times 10^3/\mu\text{L}$ (mean, 545 $\times 10^3/\mu\text{L}$) in females.³⁵ Besch-Williford presented a reference interval of platelets as 245–910 $\times 10^3/\mu\text{L}$, with a mean of 650 $\times 10^3/\mu\text{L}$ in Fitch (wild coat color) ferrets and a range of 453–545 $\times 10^3/\mu\text{L}$ in albino ferrets.¹ Female ferrets develop

TABLE 111.1 Ferret Red Blood Cell Data

Reference	Breed/ Type	Gender	RBC ($\times 10^6/\mu\text{L}$)		PCV (%)		Hgb (g/dL)		MCV (fL)		MH (pg)		MCHC (%)	
			Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range
36	NS	NS	9.98	—	—	35–51	12.8	—	—	—	—	—	—	—
35	Albino	M	10.23	7.3–12.18	55.4	44–61	17.8	16.3–18.2	—	—	—	—	—	—
		F	8.11	6.77–9.76	49.2	42–55	16.2	14.8–17.4	—	—	—	—	—	—
21	Fitch	M	—	—	43.4	36–50	14.3	12–16.3	—	—	—	—	—	—
		F	—	—	48.4	47–51	15.9	15.2–17.4	—	—	—	—	—	—
1	NS	NS (adult)	—	6.77–12.18	—	36–61	—	12.0–18.2	—	—	—	—	—	—
		Fitch (adult)	—	6.5–8.7	—	43.4–48.4	—	14.3–15.9	—	—	—	—	—	—
		Albino (adult)	—	8.11–10.23	—	49.2–55.4	—	16.2–17.8	—	—	—	—	—	—
18 ^a	NS	M	—	9.64–9.69	—	49.4–49.8	—	16.7–16.8	51.4	—	17.3	—	—	33.7–33.8
		F	—	9.3–9.34	—	48.4–48.8	—	16.2–16.3	—	52.1–52.2	17.5	—	—	33.4–33.5
18 ^b	NS	NS	—	3.6–10.0	—	30–55	—	—	—	—	—	—	—	—
13	Fitch	M	11.3	10.1–13.2	53.1	48–59	16.9	15.4–18.5	47.1	42.6–51.0	15	13.7–16.0	32	30.3–34.9
13 ^a	NS	M (10wks)	6.4 \pm 0.6	5.5–7.4	32.9 \pm 1.9	29.3–36.8	11.8 \pm 0.8	10.4–13.6	51.3 \pm 1.8	47.8–54.8	18.3 \pm 0.6	17.5–19.1	35.7 \pm 0.6	34.7–37.0
		F (10wks)	6.1 \pm 0.7	5–7	32.1 \pm 2.7	27.0–34.8	11.5 \pm 1.0	9.6–12.5	52.0 \pm 1.9	49.6–54.5	18.9 \pm 0.7	17.8–19.6	35.7 \pm 0.7	34.8–36.9
		M (12wks)	6.4 \pm 0.8	4.8–7.8	33.4 \pm 2.3	30.9–38.1	12.0 \pm 0.8	11.0–13.7	51.4 \pm 1.6	49.0–53.6	18.9 \pm 1.7	17.4–22.8	35.9 \pm 0.5	34.7–36.7
		F (12wks)	6.4 \pm 0.7	5.7–7.8	34.1 \pm 2.5	31.3–38.5	12.2 \pm 0.9	11.2–13.8	52.5 \pm 3.4	48.8–57.6	19.1 \pm 1.0	17.7–20.4	35.8 \pm 0.5	35.3–37.0
		M (14– 16wks)	8.2 \pm 0.9	6.2–9.2	39.1 \pm 4.0	29.8–43.2	14.3 \pm 1.0	12.7–15.9	47.8 \pm 2.4	44.9–53.6	17.6 \pm 1.3	16.4–20.6	36.9 \pm 2.2	35.1–42.6
		M (adult)	9.1 \pm 0.9	7.1–10.2	42.3 \pm 3.7	33.6–47.2	15.5 \pm 1.3	12.0–16.9	46.6 \pm 1.9	44.1–52.5	17.1 \pm 0.8	16.5–19.7	—	—
		F (non-estrus)	8.2 \pm 0.6	7.5–9.3	39.1 \pm 2.6	35.6–44.7	14.5 \pm 1.0	12.9–15.9	48.4 \pm 3.0	44.4–53.7	17.6 \pm 1.0	16.4–19.4	37.0 \pm 2.0	35.1–42.2
		F (in estrus)	8.3 \pm 0.5	7.5–9.3	38.8 \pm 2.5	34.6–43.3	13.4 \pm 0.9	11.9–15.0	46.8 \pm 1.3	45.2–48.7	16.2 \pm 0.3	15.8–16.8	34.5 \pm 0.6	33.2–35.3

^aData supplied by Marshall Farms.

^bReference data supplied by the University of Miami Avian Diagnostic Laboratory.

NS, not specified.

TABLE 111.2 Ferret White Blood Cell Data

Reference	Breed/ Type	Gender	WBC ($\times 10^3/\mu\text{L}$)		Neutrophils (%)		Lymphocytes (%)		Eosinophils (%)		Basophils (%)		Monocytes (%)	
			Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range
36	NS	NS	—	9–13	65	—	35	—	0	—	0	—	0	—
35	Albino	M	9.7	4.4–19.1	57	11–82	35.6	12–54	2.4	0–7	0.1	0–2	4.4	0–9
	Albino	F	10.5	4.0–18.2	59.5	43–84	33.4	12–50	2.6	0–5	0.2	0–1	4.4	2–8
21	Fitch	M	11.3	7.7–15.4	40.1	24–78	49.7	28–69	2.3	0–7	0.7	0–2.7	6.6	3.4–8.2
	Fitch	F	5.9	2.5–8.6	31.1	12–41	58	25–95	3.6	1–9	0.8	0–2.9	4.5	1.7–6.3
1	NS	NS (adult)	—	2.5–19.1	—	11–84	—	12–95	—	1–9	—	0–3	—	0–9.1
	Fitch	NS (adult)	—	5.9–11.3	—	31.1–40.1	—	49.7–58.0	—	2.3–3.6	—	0.7–0.8	—	4.5–6.6
	Albino	NS (adult)	—	9.7–10.5	—	57.0–59.5	—	33.4–35.6	—	2.4–2.6	—	0.1–0.2	—	4.4–4.6
20	NS	NS	—	2.8–8.0	—	—	35	—	—	—	—	—	—	—
18 ^a	NS	M	—	8.9–9.2	—	47–48	—	46–48	—	3.0–3.5	—	0–0.49	—	1.0–1.19
		F	—	7.0–7.6	—	49–50	45	—	—	3.0–3.3	—	0–0.34	—	1.0–1.12
18 ^b	NS	NS	—	3.3–15.9	—	9.0–54	—	34–85	—	0–10	—	0–3	—	0–8
13	Fitch	M	6.2	1.7–11.9	—	24–72	—	26–73	—	0–3	—	—	—	1–4
13 ^a	NS	M (10 wks)	8.0 \pm 2.1	5.3–12.0	32.7 \pm 5.3	24.3–45.1	54.8 \pm 5.9	42.2–64.3	4.4 \pm 1.1	2.7–6.1	0.1 \pm 0.1	0–0.2	2.8 \pm 0.8	1.7–4.3
		F (10 wks)	9.2 \pm 2.0	6.7–12.6	28.6 \pm 4.9	20.6–76.6	60.0 \pm 6.4	52.4–68.2	4.2 \pm 1.8	2.1–6.9	0.1 \pm 0.0	0–0.1	2.5 \pm 1.0	1.4–4.1
		M (12 wks)	8.4 \pm 2.0	5.3–11.7	43.3 \pm 14.2	24.3–68.3	46.1 \pm 14.1	22.1–62.8	4.4 \pm 0.9	3.3–5.8	0.1 \pm 0.3	0–1.3	2.1 \pm 1.1	0.7–4.7
		F (12 wks)	6.7 \pm 1.2	5.8–9.8	27.9 \pm 3.7	21.7–32.4	61.8 \pm 3.5	57.8–67.0	3.7 \pm 1.0	2.2–5.7	0.1 \pm 0.1	0–0.3	2.0 \pm 0.3	1.5–2.4
		M (14– 16 wks)	9.5 \pm 3.7	—	37.5 \pm 8.9	27.9–58.2	50.9 \pm 9.0	30.1–60.6	5.4 \pm 1.3	3.6–8.2	0.1 \pm 0.1	0–0.2	1.7 \pm 0.5	1.1–2.9
		M (adult)	8.4 \pm 2.5	4.9–13.8	41.5 \pm 15.4	24.0–76.6	47.4 \pm 15.3	14.7–66.6	5.6 \pm 1.5	1.9–8.5	0.1 \pm 0.1	0–0.3	1.7 \pm 1.0	0.7–5.0
		F (nonestrus)	7.2 \pm 2.3	5.1–12.6	57.7 \pm 6.8	48.8–71.0	33.3 \pm 6.1	22.7–43.3	4.3 \pm 2.1	2.3–8.5	0	0–0.1	1.8 \pm 0.7	1.0–3.0
F (in estrus)	5.7 \pm 1.5	5.2–8.2	43.2 \pm 9.4	33.1–60.9	48.2 \pm 9.6	32.9–59.1	3.1 \pm 1.2	1.6–5.6	0	0–0.1	1.6 \pm 0.5	1.1–2.7		

^aData supplied by Marshall Farms.^bReference data supplied by the University of Miami Avian Diagnostic Laboratory.

NS, not specified.

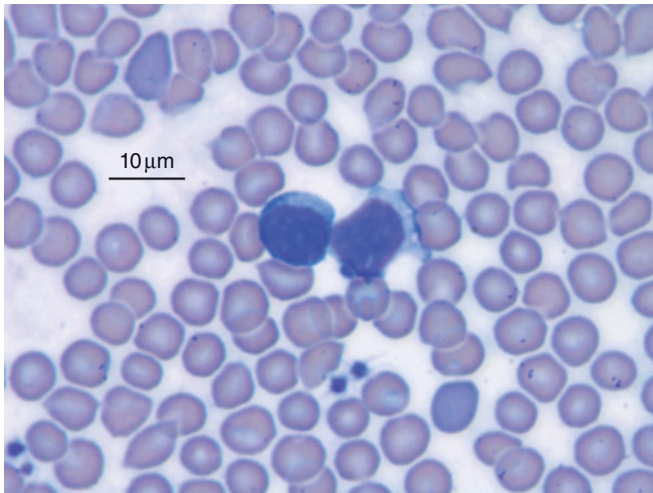


FIGURE 111.1 Blood smear from a ferret. Platelets and lymphocytes. Modified Wright's stain; 100× objective.

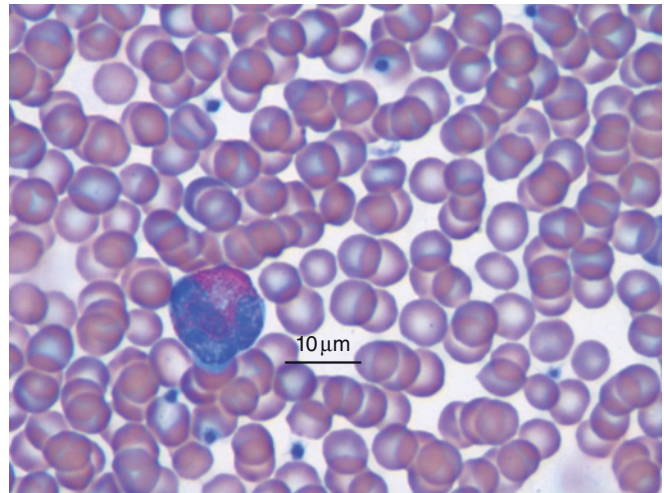


FIGURE 111.3 Blood smear from a ferret. Eosinophil. Modified Wright's stain; 100× objective.

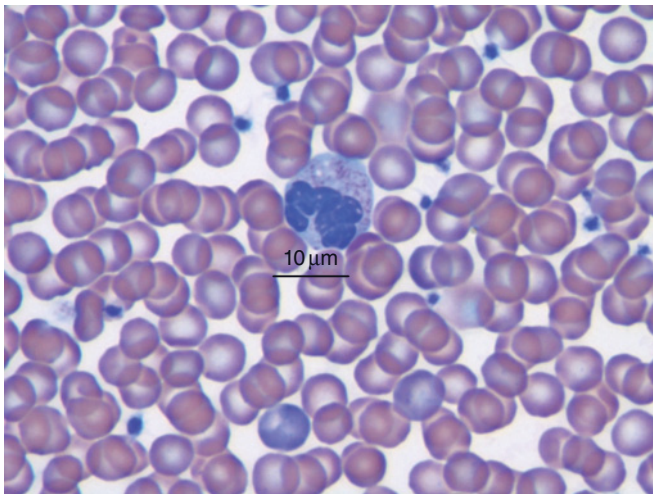


FIGURE 111.2 Blood smear from a ferret. Neutrophil. Modified Wright's stain; 100× objective.

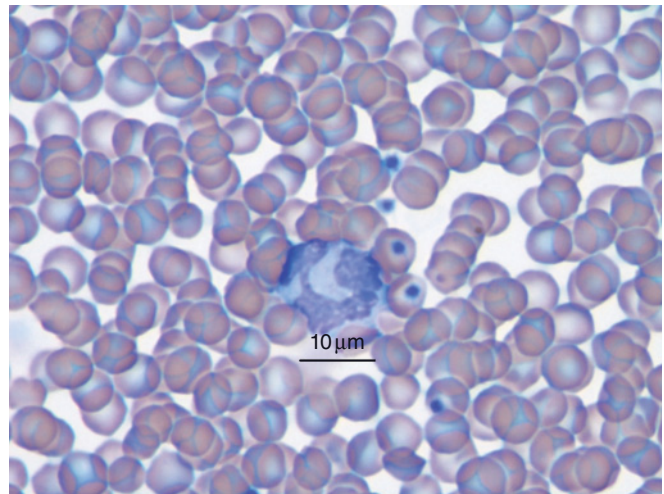


FIGURE 111.4 Blood smear from a ferret. Monocyte. Modified Wright's stain; 100× objective.

thrombocytopenia during estrus.³³ In females with severe anemia associated with hyperestrogenism, platelet counts may be less than $20 \times 10^3/\mu\text{L}$.²⁰ Blood group antigens have not been identified in the ferret and repeated transfusions do not seem to pose the same risk as in other mammals.^{15,16,31,34}

Leukocytes

Neutrophils are the predominant leukocyte observed when performing differential counts. The mean diameter of neutrophils was reported as 11.2 μm in males (range, 10–13.1 μm) and 9.6 μm in females (range, 9.2–10.0 μm).^{5,35} The cytoplasm of ferret neutrophils contains polychromatic granules (Fig. 111.2). The mean diameter of small lymphocytes was reported as 7.7 μm in males (range, 6.2–9.2 μm) and 8.7 μm in females

(range, 7.7–10.0 μm); the mean diameter of large lymphocytes was reported as 11.1 μm in males (range, 9.2–13.1 μm) and 11.9 μm in females (Fig. 111.2).³⁵ Eosinophils (mean diameter 12.7 μm in males, 12.6 μm in females) have either a one or two lobed nucleus and have more numerous and rounder granules compared to neutrophils (Fig. 111.3).^{34,35} The nucleus of basophils is segmented, and their mean diameter was reported as 13.5 μm in males and 13.8 μm in females.³⁵ Monocyte diameters were in the range 12–18 μm , and almost all cells were vacuolated (Fig. 111.4).³⁵

BONE MARROW CYTOLOGY

In normal ferrets, the myeloid to erythroid (M:E) ratio was found to be $3.4 \pm 1.1:1.0$.³³ Hypoplasia of the bone

TABLE 111.3 Ferret Coagulation Data

Parameter	Mean ± SD	Reference
Clotting time (seconds)	120 ± 0.5	22
Prothrombin time (seconds)	15.7 ± 0.4	35
	10.3 ± 0.1	22
Activated partial thromboplastin time (seconds)	18.4 ± 1.4	22

marrow in ferrets frequently occurs secondary to prolonged exposure to estrogens associated with protracted estrus.^{32,33} Severely affected jills had depletion of both erythroid and granulocytic precursors and almost a total absence of megakaryocytes.³³

OTHER HEMATOLOGIC VALUES

The RBC sedimentation rate in ferrets was reported as 1–3 mm/hour.³⁶ Because of the almost negligible RBC sedimentation rate in ferrets, blood for packed cell volume (PCV) determinations must be spun for 20% longer than samples from other species.³⁵ Observed coagulation values for ferrets are presented in Table 111.3.²²

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Hematology of the Guinea Pig

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Blood Collection
Morphology and Numbers of Peripheral Blood Cells
Erythrocytes
Platelets
Leukocytes

Bone Marrow Cytology
Other Hematologic Values

Acronyms and Abbreviations

Hgb, hemoglobin; KC, Foa-Kurloff cells; MCHC, mean corpuscular hemoglobin concentration; MCV, mean cell volume; M:E, myeloid to erythroid; PCV, packed cell volume; RBC, red blood cell; WBC, white blood cell.

BLOOD COLLECTION

Blood collection from guinea pigs is a balancing act determining the volume of blood needed for analysis, and selecting a vessel or method that will supply the required quantity without threatening the animal's well-being. Up to 50 μL of blood can be obtained by filling a single microhematocrit tube from the metatarsal veins, ear veins, saphenous veins, or toenails of non-anesthetized guinea pigs. Blood is collected after the vessels are pricked with either a sterile lancet or a sterile hypodermic needle (23–27 gauge), or the toenails cut to the “quick” with nail clippers.²⁰ Somewhat larger samples can be obtained from the saphenous veins, by first anesthetizing the guinea pig, inserting a sterile hypodermic needle into the vessel, and then collecting the blood as it drips from the hub of the needle. For research purposes, blood collection methods that yield significantly larger volumes of blood include cardiac or vena cava punctures (both generally used as a terminal procedure in anesthetized caviae), venipuncture of the jugular or saphenous veins, or collection from the retro-orbital plexus in anesthetized animals (as described for rats in Chapter 110).^{4,11,20,26}

The blood volume of the adult guinea pig is approximately 69–75 mL/kg of body weight, and about 7–10% of the blood volume (0.5–0.7 mL/100 g of body weight) can be safely drawn in a single collection from a healthy guinea pig.^{9,12,20,24}

MORPHOLOGY AND NUMBERS OF PERIPHERAL BLOOD CELLS

Erythrocytes

Guinea pig red blood cells (RBCs) have moderate anisocytosis with a diameter ranging between 6.6 and 7.9 μm , although microcytes, when present, may be 3.5 μm in diameter (Fig. 112.1).²⁸ The guinea pig RBC is the largest in comparison with the other common laboratory animal species.¹⁴ Polychromatic RBCs total approximately 25% of circulating RBCs in neonates, 4.5% in juveniles, and 1.5% in adult caviae.^{1,28} The RBC indices in guinea pigs (i.e. RBC count, hemoglobin [Hgb], and packed cell volume [PCV]) given in Table 112.1 are relatively low when compared with the values observed in other laboratory rodent species.^{18,20,30}

Platelets

Guinea pig platelets have an irregular oval shape (2–3 μm in length), and the periphery of the cytoplasm is pale in comparison to a more intensely stained central zone (Fig. 112.1).²⁸ Reference intervals for circulating platelets have been reported in several publications: $120\text{--}132 \times 10^3/\mu\text{L}$;²⁸ $161\text{--}368 \times 10^3/\mu\text{L}$;^{22,23} $530 \pm 149 \times 10^3/\mu\text{L}$;²⁷ $250\text{--}850 \times 10^3/\mu\text{L}$;⁹ $250\text{--}850 \times 10^3/\mu\text{L}$;¹⁵ $260\text{--}740 \times 10^3/\mu\text{L}$.¹²

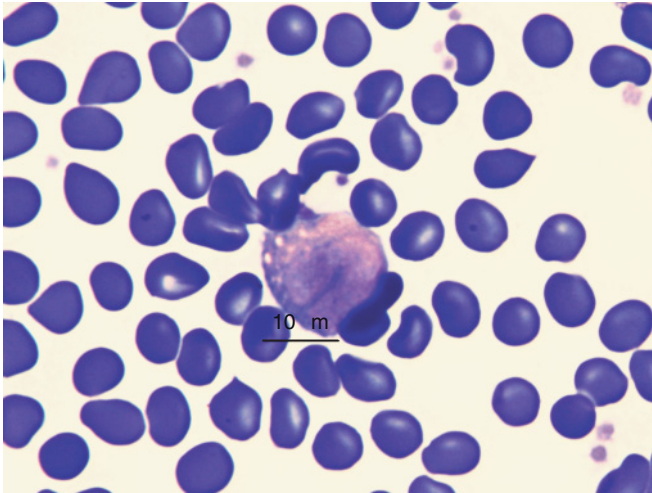


FIGURE 112.1 Guinea pig blood. Erythrocytes with moderate anisocytosis, several platelets, and a monocyte with a few cytoplasmic vacuoles; Wright's stain; 100× objective.

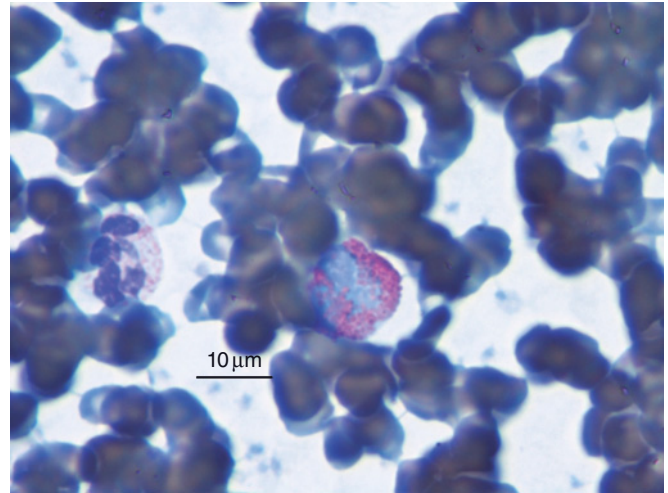


FIGURE 112.3 Guinea pig blood. Eosinophil with numerous bright eosinophilic granules filling the cytoplasm and adjacent heterophil; Wright's stain; 100× objective.

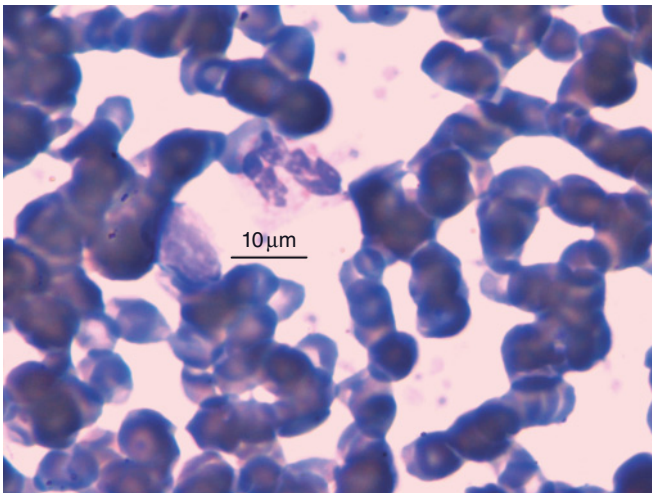


FIGURE 112.2 Guinea pig blood. Heterophil with a few small eosinophilic cytoplasmic granules and a small lymphocyte; Wright's stain; 100× objective.

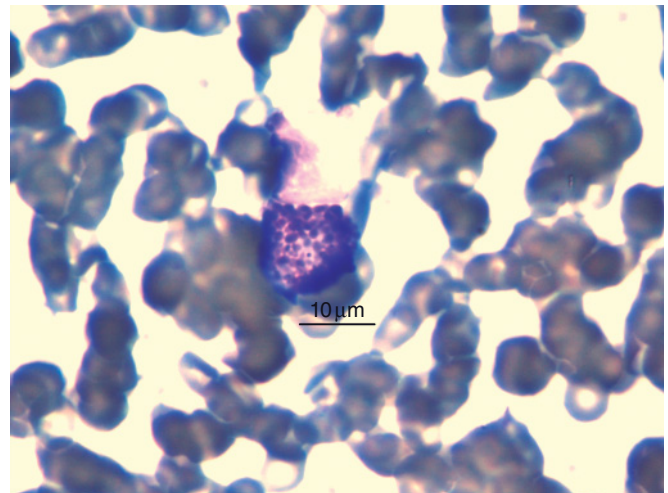


FIGURE 112.4 Guinea pig blood. Basophil with numerous oval dull basophilic-lavender granules filling the cytoplasm; Wright's stain; 100× objective.

Leukocytes

Guinea pig heterophils are the counterpart of the neutrophil in other species.²⁵ Heterophils are approximately 10–12 μm in diameter, have a condensed, segmented nucleus (with up to five or more segments), and eosinophilic granules in the cytoplasm, causing some to refer to it as a pseudoeosinophil (Fig. 112.2). A “drumstick” sex chromatin lobe may be present on the nuclei of heterophils in female guinea pigs. Cavy eosinophils are larger than heterophils (about 10–15 μm in diameter), have a less segmented nucleus with a lesser degree of pyknosis, and thick, round bright red granules that almost completely fill the cytoplasm (Fig. 112.3).²⁸

Basophils are rarely found, are about the same size as heterophils or somewhat larger, have a lobulated homogeneously purple-stained nucleus, and their cytoplasm is closely packed with round violet granules of varying size (Fig. 112.4).²⁸ Lymphocytes are the predominant leukocyte (Table 112.2), and small lymphocytes, which are present in greater numbers than large lymphocytes, are not much larger than RBCs. The small lymphocytes of cavies have a round, condensed nucleus surrounded by a narrow band of cytoplasm. Large lymphocytes are almost twice as large as small lymphocytes, with a less condensed, more oval-shaped nucleus and a brighter, broader zone of cytoplasm that may contain variably-sized azurophilic granules.²⁸ Guinea pig monocytes are

TABLE 112.1 Referenced Erythrocyte Parameters for the Guinea Pig (*Cavia porcellus*)

Reference	Gender	RBC ($\times 10^6/\mu\text{L}$)		PCV (%)		Hgb (g/dL)		MCV (fL)		MCH (pg)		MCHC (%)	
		Mean \pm S.D.	Range	Mean \pm S.D.	Range	Mean \pm S.D.	Range	Mean \pm S.D.	Range	Mean \pm S.D.	Range	Mean \pm S.D.	Range
28	NS	5.37	4.62–6.48	—	—	15.3	11.2–16.1	—	—	—	—	—	—
21	NS	5.4	—	43	—	13.4	—	81	—	25	—	30	—
22	M/F	—	5.49–8.69	—	37.5–44.2	—	11.4–13.5	—	54.6–62	—	16.5–18.8	—	26.1–34.1
27	NS	4.92 \pm 0.54	—	41.2 \pm 3.6	—	12.4 \pm 1.3	—	84.1 \pm 4.5	—	—	—	30.1 \pm 1.2	—
2	NS	—	5–8	—	32–50	—	10–16	—	50–68	—	16–22	—	30–34
3	NS	—	5–8	—	32–50	—	10–16	—	50–67	—	—	—	30–34
23	M ($n = 110$)	5.6 \pm 0.62	4.36–6.84	42 \pm 2.5	37–47	14.4 \pm 1.38	11.6–17.2	77 \pm 3	71–83	25.7 \pm 0.75	24.2–27.2	34.3 \pm 2.28	29.7–38.9
	F ($n = 95$)	4.75 \pm 1.2	3.35–6.15	45.4 \pm 2.25	40.9–49.9	14.2 \pm 1.42	11.4–17	91 \pm 2.45	86.1–95.9	25.7 \pm 0.8	23.1–26.3	31.3 \pm 1.55	28.2–34.4
29	NS	—	4–7	—	35–45	—	11–17	—	—	—	—	—	—
14	M (2–30 d)	4.67 \pm 0.65	—	38.3 \pm 4.5	—	11.63 \pm 1.5	—	82.4 \pm 4.0	—	—	—	29.7 \pm 1.4	—
	F (2–30 d)	4.58 \pm 0.52	—	42.9 \pm 2.9	—	11.07 \pm 1.2	—	82.1 \pm 4.6	—	—	—	29.5 \pm 1.1	—
	M (31–60 d)	5.18 \pm 0.47	—	42.9 \pm 2.9	—	13.06 \pm 1.0	—	82.9 \pm 3.9	—	—	—	30.3 \pm 0.9	—
	F (31–60 d)	5.19 \pm 0.4	—	43.5 \pm 3.1	—	13.3 \pm 0.8	—	84.0 \pm 4.7	—	—	—	30.7 \pm 1.0	—
	M (63–90 d)	5.64 \pm 0.38	—	46.3 \pm 2.3	—	14.04 \pm 0.9	—	82.2 \pm 2.7	—	—	—	30.3 \pm 1.2	—
	F (63–90 d)	5.52 \pm 0.35	—	46.2 \pm 2.8	—	14.2 \pm 0.9	—	83.7 \pm 1.9	—	—	—	30.8 \pm 1.6	—
	M (4–6 mo)	5.81 \pm 0.62	—	45.1 \pm 4.5	—	14.07 \pm 1.0	—	77.7 \pm 3.8	—	—	—	31.2 \pm 1.2	—
	F (4–6 mo)	5.27 \pm 0.49	—	44.1 \pm 3.8	—	13.55 \pm 1.4	—	83.7 \pm 3.1	—	—	—	30.7 \pm 1.0	—
	M (7–12 mo)	5.55 \pm 0.51	—	44.0 \pm 3.7	—	13.9 \pm 1.4	—	79.4 \pm 3.7	—	—	—	31.6 \pm 1.1	—
	F (7–12 mo)	4.87 \pm 0.24	—	41.2 \pm 2.4	—	12.4 \pm 0.7	—	84.6 \pm 3.0	—	—	—	30.1 \pm 0.9	—
	M (13–28 mo)	5.37 \pm 0.46	—	43.9 \pm 3.7	—	13.56 \pm 1.1	—	81.8 \pm 3.5	—	—	—	30.9 \pm 1.4	—
	F (13–28 mo)	4.67 \pm 0.39	—	39.8 \pm 2.6	—	11.76 \pm 0.8	—	85.4 \pm 4.5	—	—	—	29.6 \pm 0.8	—
9	NS	—	4.5–7.0	—	37–48	—	11–15	—	—	—	—	—	—
15	NS	—	4.5–7.0	—	37–48	—	11–15	—	—	—	—	—	—
12	NS	—	3.2–8.0	—	32–50	—	10–17.2	—	71–96	—	23–27	—	26–39

NS, not specified.

TABLE 112.2 Referenced Leukocyte Parameters for the Guinea Pig (*Cavia porcellus*)

Reference	Gender	WBC ($\times 10^3/\mu\text{L}$)		Heterophils (%)		Lymphocytes (%)		Eosinophils (%)		Basophils (%)		Monocytes (%)	
		Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range
28	NS	9	3.2–15	—	18–35	—	55–75	—	1–5	—	0–3	—	3–12
21	NS	9.9	—	38	—	55	—	3.5	—	0.3	—	2.7	—
22	M/F	—	7.8–20.7	—	21.7–47.7	—	41.3–68.5	—	2.1–7.8	—	0.6–2.7	—	2.46–5.84
27	NS	11.2 \pm 2.85	—	—	—	—	—	—	—	—	—	—	—
2	NS	—	11–22	—	28–47	—	39–52	—	0–11	—	0–2	—	2–10
3	NS	—	10–14	—	28–47	—	39–60	—	1–11	—	0–2	—	2–10
23	M ($n = 84$)	11.5 \pm 3	5.5–17.5	42 \pm 7	28–56	49 \pm 6.75	40–62.5	4.0 \pm 1.5	1–7	0.7 \pm 0.5	0–1.7	4.3 \pm 0.5	3.3–5.3
	F ($n = 80$)	10.8 \pm 2.8	5.2–16.4	31.1 \pm 5.4	20.3–41.9	63.4 \pm 8.5	46.4–80.4	3.5 \pm 1.75	0–7	0.2 \pm 0.3	0.0.8	1.8 \pm 0.4	1.0–2.6
29	NS	—	7–14	—	20–60	—	30–80	—	0–5	—	0–1	—	2–20
14	M (2–30 d)	3.73 \pm 0.94	—	27.9 \pm 10.8	—	70.7 \pm 11.7	—	2.2 \pm 2.1	—	0.22 \pm 0.48	—	2.1 \pm 1.9	—
	F (2–30 d)	4.09 \pm 1.0	—	21.2 \pm 6.4	—	74.9 \pm 7.7	—	1.9 \pm 1.7	—	0.13 \pm 0.4	—	1.4 \pm 1.4	—
	M (31–60 d)	5.52 \pm 1.8	—	29.0 \pm 10.7	—	66.2 \pm 13.6	—	1.0 \pm 0.8	—	0.13 \pm 0.28	—	2.1 \pm 1.5	—
	F (31–60 d)	7.04 \pm 2.01	—	25.8 \pm 11.7	—	71.5 \pm 12.5	—	1.0 \pm 0.8	—	0.08 \pm 0.21	—	1.6 \pm 1.5	—
	M (63–90 d)	5.94 \pm 1.2	—	31.9 \pm 10.7	—	65.9 \pm 10.6	—	0.6 \pm 0.6	—	0.19 \pm 0.39	—	1.3 \pm 1.1	—
	F (63–90 d)	7.98 \pm 2.3	—	26.3 \pm 7.1	—	70.5 \pm 7.1	—	1.4 \pm 1.2	—	0.1 \pm 0.2	—	1.7 \pm 2.0	—
	M (4–6 mo)	9.58 \pm 3.17	—	20.8 \pm 6.1	—	75.3 \pm 6.7	—	1.2 \pm 1.0	—	0.19 \pm 0.24	—	1.9 \pm 1.4	—
	F (4–6 mo)	10.24 \pm 1.87	—	24.3 \pm 11.3	—	71.3 \pm 11.9	—	2.0 \pm 1.8	—	0.31 \pm 0.47	—	2.2 \pm 1.6	—
	M (7–12 mo)	11.5 \pm 2.0	—	23.2 \pm 5.1	—	71.4 \pm 4.0	—	2.6 \pm 4.5	—	0	—	2.8 \pm 1.4	—
	F (7–12 mo)	10.93 \pm 3.2	—	23.5 \pm 11.0	—	71.4 \pm 11.3	—	2.3 \pm 2.5	—	0.08 \pm 0.18	—	2.7 \pm 2.2	—
	M (13–28 mo)	13.53 \pm 2.5	—	30.3 \pm 15.7	—	64.8 \pm 16.1	—	2.1 \pm 2.4	—	0.18 \pm 0.24	—	2.7 \pm 1.6	—
F (13–28 mo)	9.88 \pm 2.1	—	24.7 \pm 10.6	—	69.4 \pm 13.2	—	2.3 \pm 2.1	—	0.22 \pm 0.29	—	3.4 \pm 3.6	—	
9	NS	—	7–18	—	28–44	—	39–72	—	1–5	—	0–3	—	3–12
15	NS	—	7–18	—	28–44	—	39–72	—	1–5	—	0–3	—	3–12
12	NS	—	5.5–17.5	—	22–48	—	39–72	—	0–7	—	0–2.7	—	1–10

NS, not specified.

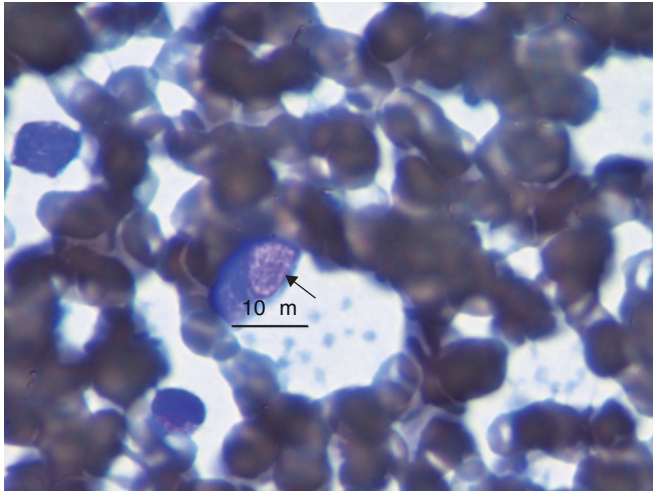


FIGURE 112.5 Guinea pig blood. Kurloff cell with pale basophilic 8 μ m cytoplasmic inclusion (arrow) displacing nucleus; Wright's stain; 100 \times objective.

usually larger than the large lymphocytes, have an oval nucleus with a dispersed chromatin structure, and have a gray-blue cytoplasm that is darker than the cytoplasm of large lymphocytes (Fig. 112.1).

Occasional Foa-Kurloff cells (KC), which are unique to the guinea pig, may be observed in the circulation, accounting for up to 3–4% of the differential leukocyte count.^{14,18} There is no significant difference in numbers of KC observed in males and females after 2–3 months of age.¹⁴ The KC cell is a specialized mononuclear leukocyte that contains an intracytoplasmic, finely granular to fibrillar 1–8 μ m inclusion body consisting of a mucopolysaccharide (Fig. 112.5).^{20,25} The inclusion is within a cytoplasmic vacuole and displaces the nucleus.²⁵ Whereas these cells may be found in blood vessels and in thymus, their highest density shifts from lungs and red pulp of the spleen to thymus and placenta under the stimulation of estrogen and pregnancy.¹³ The exact origin and function of these cells is unknown, although it has been speculated that they may function as killer cells in the general circulation or as protectors of fetal antigen in the placenta.^{5,8,19}

BONE MARROW CYTOLOGY

Bone marrow is perhaps best obtained from the proximal end of the femur in guinea pigs. The myeloid to erythroid (M:E) ratio was estimated to be between 1.2:1.0 and 1.6:1.0.^{6,7,10,20,24}

OTHER HEMATOLOGIC VALUES

The sedimentation rate for blood from guinea pigs was reported as follows: 2.3–8.1 mm/hr;^{22,23} 1–14 mm/hr;³ 2–4 mm/hr;²⁹ and 1.1–14 mm/hr.¹² Observed coagulation values for guinea pigs¹⁷ are presented in Table 112.3.

TABLE 112.3 Observed Coagulation Values in Guinea Pigs

Parameter	Mean \pm SD	Reference
Bleeding time (minutes)		
Foreleg	4	17
Hindleg	4.5	17
Paw	3.5	17
Clotting time (minutes)	3 (\pm 0.7)	17
Prothrombin time (seconds)	17.6	18
	26 (\pm 2.5)	17
Activated partial thromboplastin time (seconds)	16.8 (range 13.0–22.9)	18
	28.7 (\pm 3.8)	17

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Hematology of the Mongolian Gerbil

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Blood Collection
Morphology and Numbers of Peripheral Blood Cells
Erythrocytes
Platelets
Leukocytes

Bone Marrow Cytology
Other Hematologic Values

Acronyms and Abbreviations

Hct, hematocrit; Hgb, hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; M:E, myeloid to erythroid ratio; PCV, packed cell volume; RBC, red blood cell; WBC, white blood cell.

BLOOD COLLECTION

The blood volume of the Mongolian gerbil (*Meriones unguiculatus*) is approximately 7.7 mL/100 g of body mass, and thus approximately 0.8 mL of blood can be safely collected at one time without adversely affecting the animal's well-being.⁸ In research laboratories, blood is usually collected from anesthetized gerbils from the retro-orbital venous plexus or by cardiac puncture (described for the rat in Chapter 110). Less invasive procedures, that yield significantly smaller volumes of blood, involve filling a single microhematocrit tube with blood obtained from a cut toenail or the saphenous vein after puncture with a sterile lancet or hypodermic needle. Analysis of blood samples obtained from the tail vein or heart demonstrated almost identical results for packed cell volume (PCV), hemoglobin (Hgb), and red blood cell (RBC) numbers, whereas samples obtained from the tail vein had higher white blood cell (WBC) counts associated with a greater concentration of lymphocytes.¹⁷ Lipemic plasma, prevalent in both sexes and at all ages, with a more pronounced lipemia in males 13 months of age or older, was attributed to addition of sunflower seeds to the diet.¹⁷

MORPHOLOGY AND NUMBERS OF PERIPHERAL BLOOD CELLS

Reported hematologic values for the gerbil are provided in Tables 113.1 and 113.2. A significant sexual dimorphism exists with respect to RBC indices (i.e.

mean corpuscular volume [MCV], Hgb, hematocrit [Hct], and mean corpuscular hemoglobin concentration [MCHC]), with higher values for the erythrocyte indices reported in adult male gerbils.^{2,4,5,9,11,13,20}

Erythrocytes

When compared to adult reference values, neonates have RBC macrocytosis, panleukocytosis, and lower RBC counts. These values reach adult values by about 8 weeks of age.^{19,20} Polychromasia/reticulocytosis and basophilic stippling are notable features of gerbil blood, particularly of newborns.¹⁵ Basophilic stippling and polychromasia of RBCs were observed in 40% of cells of fetal and newborn gerbils, but declined to adult levels of $5.4 \pm 2.4\%$ by 20 weeks of age.^{15,18} A range of 5–40 basophilic particles, approximately 0.3 μm in diameter, may be present in the stippled RBCs.¹⁷ Red blood cells with diffuse basophilic staining usually are larger than the mature RBCs (Fig. 113.1). Red blood cells with basophilic stippling are thought to be relatively immature erythrocytes that still possess remnants of cytoplasmic ribonucleoprotein.^{6,18} Normochromic stippled RBCs may be slightly larger than mature erythrocytes, but are smaller than the diffusely stained basophilic RBCs. The presence of stippled RBCs should not be confused with the intracellular organism *Mycoplasma* (*Haemobartonella*) sp. In another study, polychromatophilic RBCs were found with almost equal frequency in male and female gerbils (about 1.7%).² The presence of stippled RBCs and reticulocytes at levels higher than observed in most other domestic

TABLE 113.1 Referenced Erythrocyte Parameters of the Mongolian Gerbil (*Meriones unguiculatus*)

Reference	Gender	RBC ($\times 10^6/\mu\text{L}$)		PCV (%)		Hgb (g/dL)		MCH (pg)		MCV (fL)		MCHC (%)	
		Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range
17	M (2mo)	8.1	7.0–8.9	47	41–51	13.9	12.1–15.4	—	—	—	—	—	—
	M (7mo)	8.3	7.8–8.9	47	44–50	14.4	13.4–15.6	—	—	—	—	—	—
	M (13mo)	8.1	7.1–8.6	46	42–49	15.2	13.1–17.9	—	—	—	—	—	—
	F (2mo)	7.7	7.3–8.2	45	42–47	13	12.1–13.8	—	—	—	—	—	—
	F (7mo)	8.7	7.6–9.9	46	43–50	13.7	12.4–15.2	—	—	—	—	—	—
	F (13mo)	8.6	8.0–9.4	47	43–50	14.4	13.1–16.9	—	—	—	—	—	—
11	—	—	7–8	—	37–47	—	14–16	—	—	—	—	—	—
12	NS	8.849 \pm 0.509	7.87–9.97	—	—	—	—	17.49 \pm 1.089	16.13–19.40	54.46 \pm 3.72	46.64–60.04	32.14 \pm 0.71	30.64–33.33
	M	—	—	49.25 \pm 2.03	46–52	15.88 \pm 0.57	15.2–16.8	—	—	—	—	—	—
	F	—	—	46.80 \pm 1.4	44–48	15.00 \pm 0.39	14.4–15.6	—	—	—	—	—	—
2	M	—	—	47.5 \pm 0.978	44–49	14.75 \pm 0.44	13.8–16.2	—	—	—	—	—	—
	F	—	—	45.8 \pm 1.32	43–49	14.14 \pm 0.51	13.5–14.8	—	—	—	—	—	—
16	M (3mo)	8.9	—	47.4	—	15.9	—	17.1	—	54.5	—	32.4	—
13	NS	—	7.87–9.97	—	46–52	—	15.2–16.8	—	16.1–19.4	—	46.6–60	—	30.6–33.3
21	NS	—	7–8	—	35–45	—	14–16	—	16.3–19.40	—	46.64–60.04	—	30.64–33.33
3	NS	8.5	7.0–10	48	41–52	15	12.1–16.9	—	—	—	—	—	—
20	NS	8.5	7–10	—	35–50	—	10–17	—	—	—	—	—	—
8	NS	—	8–9	—	43–49	—	12.6–16.2	—	—	—	—	—	—
10	NS	8.5	7–10	48	41–52	15	12.6–16.2	—	—	—	—	—	—

NS, not specified.

TABLE 113.2 Referenced Leukocyte Parameters of the Mongolian Gerbil (*Meriones unguiculatus*)

Reference	Gender	WBC ($\times 10^3/\mu\text{L}$)		Neutrophils (%)		Lymphocytes (%)		Monocytes (%)		Eosinophils (%)		Basophils (%)	
		Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range
17	M (2 mo)	9.8	4.7–15.0	19.3	14.8–38	75.5	68–76.7	3	2.1–3.3	2	0–2.5	0.7	0–1.3
	M (7 mo)	11.2	5.1–15.9	14.2	9.8–16.3	83	80.5–86.2	2.7	0–3.2	0.09	0–1.9	0.53	0–1.2
	M (13 mo)	9.1	4.3–12.3	18.7	9.3–23.6	75.8	68–76.8	3.3	0–6.5	1.1	0–1.6	0.88	0–1.6
	F (2 mo)	8.3	4.5–15.4	20.5	17.8–38.3	74.7	68.9–76	2.4	2.2–5.8	1.2	0–1.8	0.84	0–1.3
	F (7 mo)	9.9	4.7–16.7	16.2	6.4–22.8	79.8	78.4–85.1	2	0–4.2	1	0–2.4	0.6	0–1.2
	F (13 mo)	9.5	5.6–12.8	21	10.7–25.8	74.8	58.9–78.1	3.1	1.7–6.2	1	0–2.3	0.42	0–0.8
12	M	13.532 \pm 5.85	6.506–21.600	13.9 \pm 6.75	2–23	84.8 \pm 7.90	73–97	—	—	—	—	—	—
	F	8.696 \pm 0.415	7.509–10.900	23.4 \pm 12.23	7–41	74.8 \pm 12.36	58–92	—	—	—	—	—	—
	NS	—	—	—	—	—	—	0.3	0–3	1.1	0–4	0.05	0–1
2	M	12.1 \pm 1.99	8.64–15.4	20.2	7.8–33.5	78.2	—	0.8	—	1.2	—	1.5–1.1	—
	F	9.65 \pm 1.72	7.34–14.6	26.2	17.1–35.7	72.8	—	1.4	—	1.36	—	1.1	—
16	M (3 mo)	12.4	—	15.6	—	80.6	—	0.2	—	1.1	—	0.7	—
13	NS	—	6.51–21.6	—	2–23	—	73–97	—	0–3	—	0–4	—	0–1
21	NS	—	7.5–10.9	22	—	75	—	—	0–4	—	0–3	—	0–1
3	NS	11	4.3–21.6	19	3–41	78	32–97	3	0–9	1	0–4	0.6	0–2
20	NS	—	4.3–22	—	2–41	—	58–98	—	0–3	—	0–4	—	0–1
8	NS	—	7–15	—	5–34	—	60–95	—	0–3	—	0–4	—	0–3
10	NS	11	4.3–21.6	29.9	5–34	73.5	60–95	—	0–3	—	0–4	—	0–1

NS, not specified.

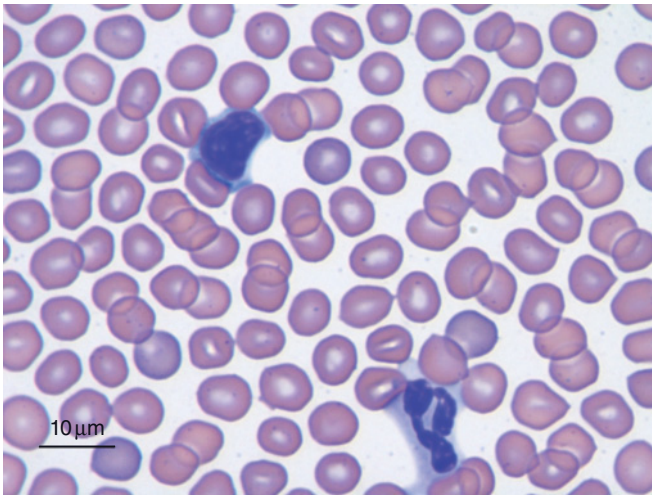


FIGURE 113.1 Blood smear from a Mongolian gerbil (*Meriones unguiculatus*). Erythrocytes with polychromasia and basophilic stippling and a lymphocyte and neutrophil. Wright’s stain; 100× objective.

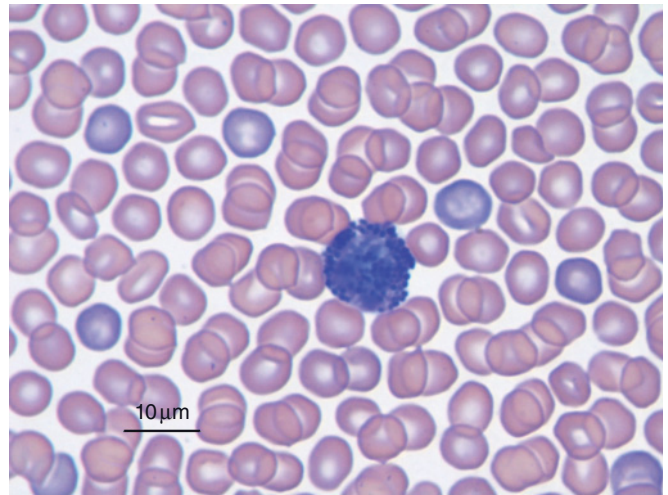


FIGURE 113.2 Blood smear from a Mongolian gerbil (*Meriones unguiculatus*). Erythrocytes and a basophil. Wright’s stain; 100× objective.

TABLE 113.3 Platelet Counts in Mongolian Gerbils (*Meriones unguiculatus*)

Age (months)	N	Gender	Platelets ($\times 10^3/\mu\text{L}$)	
			Mean	Range
2	3	M	557.66	543–575
2	3	F	794.16	767.5–830
7	3	M	608.36	540–668.8
7	3	F	669.60	603.8–720
13	3	M	609.17	432–710
13	3	F	590.67	540–632

rodent species may be a result of the relatively short RBC lifespan in gerbils, noted as 9–10 days, which necessitates continuous replacement of circulating RBCs.^{1,8,15,17}

Platelets

Platelet counts for gerbils are similar to those found in rats.¹⁷ Observed ranges of numbers of circulating platelets have been reported in several references: $638 \times 10^3/\mu\text{L}$;³ $400 \times 10^3/\mu\text{L}$ to $600 \times 10^3/\mu\text{L}$;²⁰ and $400 \times 10^3/\mu\text{L}$ to $600 \times 10^3/\mu\text{L}$.¹⁰ A comparison of platelet counts by age and gender is provided in Table 113.3.

Leukocytes

The ratio of lymphocytes to neutrophils averages 6.1:1 in male gerbils and 3.2:1 in females (Fig. 113.1).¹² The total leukocyte count in gerbils is closer to that of the mouse than the hamster.⁷ Basophils are rare but have been seen in association with nematodiasis (Fig. 113.2).¹⁴ Examples of eosinophils and monocytes are shown in Figures 113.3 and 113.4. Reported leukocyte reference

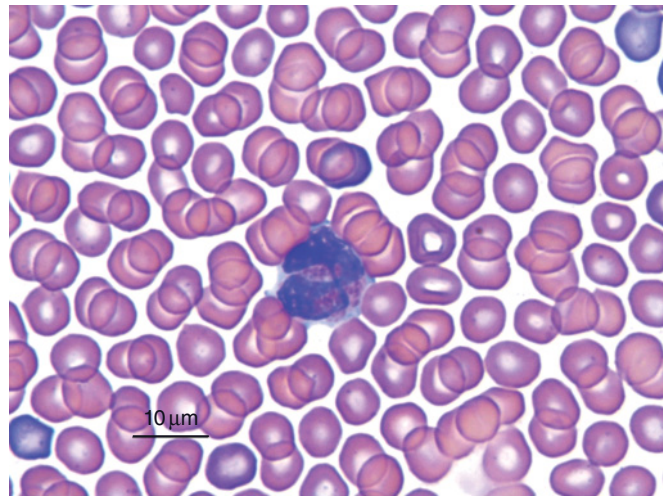


FIGURE 113.3 Blood smear from a Mongolian gerbil (*Meriones unguiculatus*). Erythrocytes and an eosinophil. Wright’s stain; 100× objective.

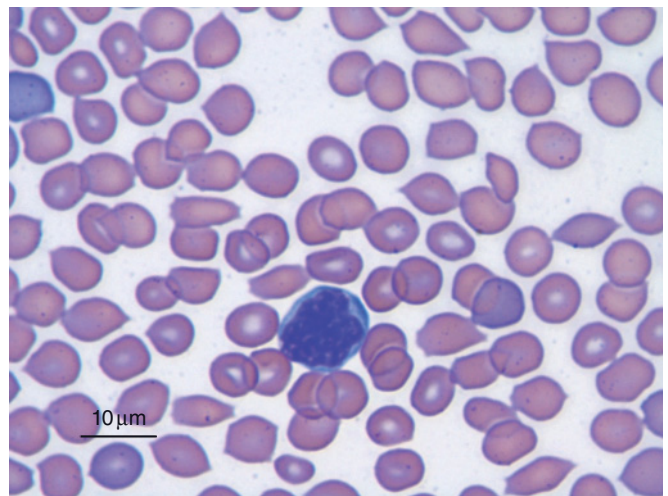


FIGURE 113.4 Blood smear from a Mongolian gerbil (*Meriones unguiculatus*). Erythrocytes and a monocyte. Wright’s stain; 100× objective.

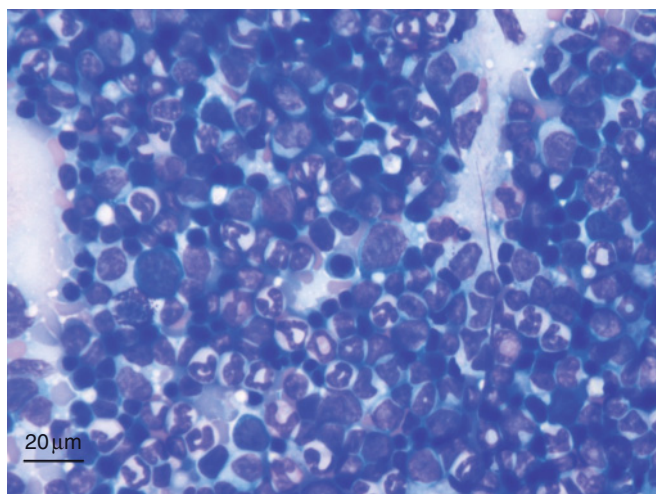


FIGURE 113.5 Bone marrow from a Mongolian gerbil (*Meriones unguiculatus*). Wright's stain; 100× objective.

intervals for Mongolian gerbils are provided in Table 113.2.

BONE MARROW CYTOLOGY

The myeloid to erythroid (M:E) ratio in the bone marrow of gerbils was reported to be $1.6 \pm 0.75:1$, with an observed range of 0.6:1 to 3.6:1.²² One study reported mean percentages of selected bone marrow cells as follows: neutrophil, 38.5%; erythroid cells, 26.8%; plasma cells, 2.0%; eosinophils, 1.9%; and basophils, 0.2%.¹⁶ Basophilic stippling was seen in $26.1 \pm 7.6\%$ of RBCs in bone marrow (Fig. 113.5).¹⁸ In another study, lymphocytes in bone marrow samples accounted for about $8.4 \pm 4.2\%$ of cells observed, with a range between 1.4% and 20.1%.²² In this study there was a significant difference between males and females in the total number of neutrophils (42% and 35%, respectively).²² Ring neutrophils are seen in gerbil marrow, similar to those observed in rats and mice.²² The bone marrow of adult gerbils contains a higher proportion of stippled red cells than is observed in the blood.¹⁸

OTHER HEMATOLOGIC VALUES

The sedimentation rate for normal gerbil blood was reported as 0–2 mm/hr.²¹

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Hematology of the Syrian (Golden) Hamster

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Blood Collection

Morphology and Numbers of Peripheral Blood Cells

Erythrocytes

Platelets

Leukocytes

Bone Marrow Cytology

Other Blood Findings

Other Hematologic Values

Acronyms and Abbreviations

Hgb, hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean cell volume; M:E ratio, myeloid to erythroid ratio; PCV, packed cell volume; RBC, red blood cell; WBC, white blood cell.

BLOOD COLLECTION

Blood is collected from laboratory hamsters either from the retro-orbital venous plexus or by cardiac puncture while the animal is anesthetized (these procedures are described for the rat in Chapter 110). Alternative blood collection methods for hamsters include use of a micro-hematocrit tube to obtain blood from a clipped toenail or from the saphenous vein after puncture with a sterile lancet or hypodermic needle. The blood volume in a hamster is between 65 and 80 mL/kg of body mass, of which approximately 1–1.5 mL can be withdrawn safely during a single collection.^{8,9}

MORPHOLOGY AND NUMBERS OF PERIPHERAL BLOOD CELLS

Reported hematologic values for the Syrian hamster (*Mesocricetus auratus*) are listed in Tables 114.1 and 114.2. Additional published hematologic reference values for other species of hamsters can be found in the literature (Table 114.3).^{1,14,17,22}

Erythrocytes

Hamster red blood cells (RBCs) are biconcave, have an average diameter of 6 μm (range, 5–7 μm), with moderate polychromasia and anisocytosis (Fig. 114.1).¹⁸ The proportion of nucleated RBCs may be as high as 10–30% in newborn hamsters, whereas in adults there are usually 2% or fewer of these cells.^{5,8} The relatively high reported reticulocyte percentages (2.0–12.5% and

0.4–2.8%) are probably due to the relatively short RBC lifespan of 50–70 days.^{18,20,21} The hematocrit and hemoglobin (Hgb) levels in neonates increase until about 8–9 weeks of age.⁵ Elevated Hgb levels are observed in 2- to 3-week-old hamsters and in animals that are anorexic or have been starved.⁹ The number of circulating RBCs decreases by 25–30% after castration of male hamsters,²¹ and testosterone administration restores RBCs to pre-castration levels.¹⁰ Red blood cell counts and Hgb concentrations are higher in hibernating animals, with the mean number of RBCs increasing from $7.7\text{--}10^6/\mu\text{L}$ to approximately $8.2 \times 10^6/\mu\text{L}$, and Hgb increasing from a mean of 13.5 g/dL to approximately 16.7 g/dL.²⁰ Red blood cell senescence is delayed and RBC destruction is virtually absent during hibernation, with an observed increase in RBC lifespan of up to 160 days, as compared with the normal erythrocyte lifespan of 50–70 days in non hibernating hamsters.^{2,19} However, no change in mean cell volume (MCV) was observed in cold acclimated hamsters as has been reported in rats.⁶

Platelets

Platelets appear to be amorphous veils of a gray-blue ground substance with violet granulation (Fig. 114.1).²⁰ Platelet numbers have been reported in several references: $336 \times 10^3/\mu\text{L}$ to $587 \times 10^3/\mu\text{L}$,²⁰ $247 \times 10^3/\mu\text{L}$ to $372 \times 10^3/\mu\text{L}$,⁵ $300 \times 10^3/\mu\text{L}$ to $573 \times 10^3/\mu\text{L}$,¹⁶ $200 \times 10^3/\mu\text{L}$ to $590 \times 10^3/\mu\text{L}$,²⁵ $200 \times 10^3/\mu\text{L}$ to $500 \times 10^3/\mu\text{L}$,⁸ $200 \times 10^3/\mu\text{L}$ to $500 \times 10^3/\mu\text{L}$,¹¹ and $297 \times 10^3/\mu\text{L}$ to $439 \times 10^3/\mu\text{L}$.¹³ Platelet counts were found to be reduced during hibernation and cold acclimation.^{6,19}

TABLE 114.1 Referenced Erythrocyte Parameters of the Syrian (Golden) Hamster (*Mesocricetus auratus*)

Reference	Gender	RBC ($\times 10^6/\mu\text{L}$)		PCV (%)		Hb (g/dl)		MCV (fL)		MCH (pg)		MCHC (%)	
		Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range
20	NS	7	6–9	—	—	14.88	—	—	—	—	—	—	—
5	NS	7.5 \pm 2.4	—	52.5 \pm 2.3	—	16.8 \pm 1.2	—	71.2 \pm 3.2	—	—	—	—	—
1	NS ($n = 23$)	6.8 \pm 0.3	—	—	—	16.0 \pm 0.3	—	73.9 \pm 3.0	—	—	—	31.6 \pm 2.1	—
14	NS	7.2	—	46	—	14.8	—	64	—	21	—	32	—
22	NS	7.5 \pm 2.4	—	52.5 \pm 2.3	—	16.8 \pm 1.2	—	71.2 \pm 3.19	—	22.3 \pm 1.27	—	32.0 \pm 2.23	—
15	M/F	—	3.96–10.3	—	32.9–58.8	—	13.1–19.2	—	64.0–77.6	—	19.9–25.8	—	27.8–37.4
16	M ($n = 84$)	7.5 \pm 1.40	4.7–10.3	52.5 \pm 2.3	47.9–57.1	16.8 \pm 1.2	14.4–19.2	70.0 \pm 3.19	64.8–77.6	22.4 \pm 1.27	19.9–24.9	32.0 \pm 2.23	27.5–36.5
	F ($n = 80$)	6.96 \pm 1.50	3.96–9.96	49.0 \pm 4.9	39.2–58.8	16.0 \pm 1.45	13.1–18.9	70.0 \pm 3.0	64.0–76.0	23.0 \pm 1.4	20.2–25.8	32.6 \pm 2.4	27.8–37.4
26	NS	—	7–8	—	45–49.8	—	16.6–18.6	—	—	—	—	—	—
25	NS	8	4–10	—	36–59	—	9.7–19	—	—	—	—	—	—
8	NS	—	6–10	—	36–55	—	10–16	—	—	—	—	—	—
11	NS	—	5–10	—	36–55	—	10–16	—	—	—	—	—	—
13	NS	7.1 \pm 0.2	—	42 \pm 1.9	—	15.2 \pm 0.6	—	59 \pm 1.0	—	21 \pm 0.8	—	36 \pm 0.8	—

NS, not specified.

TABLE 114.2 Referenced Leukocyte Parameters of the Syrian (Golden) Hamster (*Mesocricetus auratus*)

Reference	Gender	WBC ($\times 10^3/\mu\text{L}$)		Neutrophils (%)		Lymphocytes (%)		Eosinophils (%)		Basophils (%)		Monocytes (%)	
		Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range
20	NS	6.2	3.4–7.6	—	3–43	—	50–96	—	0–2	—	0	—	0–1
5	NS	7.62 \pm 1.3	—	29.9 \pm 8.0	—	73.5 \pm 9.4	—	1.1 \pm 0.0	—	0	—	2.5 \pm 0.8	—
14	NS	6.3	—	27	—	68	—	1.1	—	0	—	2.9	—
22	NS	7.62 \pm 1.3	—	21.9 \pm 5.5	—	73.5 \pm 9.4	—	1.1 \pm 0.02	—	—	—	2.5 \pm 0.8	—
15	M/F	—	5.02–10.6	—	17.1–35.2	—	50.9–92.3	—	0.22–1.54	—	0–5	—	0.4–4.4
16	M ($n = 84$)	7.62 \pm 1.3	5.02–10.2	22.1 \pm 2.5	17.1–27.1	73.5 \pm 9.4	54.7–92.3	0.9 \pm 0.32	0.26–1.54	1.0 \pm 2.0	0–5	2.5 \pm 0.8	0.9–4.1
	F ($n = 80$)	8.56 \pm 1.54	6.48–10.6	29.0 \pm 3.12	22.8–35.2	67.9 \pm 8.52	50.9–84.9	0.7 \pm 0.24	0.22–1.18	0.5 \pm 0.7	0–2.1	2.4 \pm 1.0	0.4–4.4
26	NS	—	5–23	—	10–50	—	50–70	—	0–5	—	0–1	—	0–10
25	NS	—	3–15	—	10–43	—	50–95	—	0–4.5	—	0–1	—	0–3
8	NS	—	3–11	—	10–42	—	50–95	—	0–4.5	—	0–1	—	0–3
11	NS	—	6.3–8.9	—	10–42	—	50–95	—	0–4.5	—	0–1	—	0–3
13	NS	4.7 \pm 0.8	—	24 \pm 9	—	74 \pm 9	—	0	—	0	—	2 \pm 1	—

NS, not specified.

TABLE 114.3 Referenced Ranges of Hematologic Values of Other Hamster Species

Parameter	European Hamster (<i>Cricetus cricetus</i>)		Chinese Hamster (<i>Cricetulus griseus</i>)
	Ref. 19	Ref. 20	Ref. 17
RBC ($\times 10^6/\mu\text{L}$)	6.04–9.10	6.4–8.8	4.4–9.1
PCV (%)	44–49		36.5–47.7
Hemoglobin (g/dL)	13.4–15.5	12.4–15.9	10.7–14.1
MCV (fL)	58.7–71.4		53.5–19.1
MCH (pg)	18.6–22.5		15.5–19.1
MCHC (g/dL)	26.4–32.5		27–32
WBC ($\times 10^3/\mu\text{L}$)	3.4–7.6		2.7–9.6
Neutrophils (%)	3.5–41.6		14.8–23.6
Lymphocytes (%)	50–95		68.1–84.8
Eosinophils (%)	0.0–2.1		0.3–3.1
Basophils (%)	0.0–0.2		0.0–0.5
Monocytes (%)	0.0–1.0		0.0–2.4

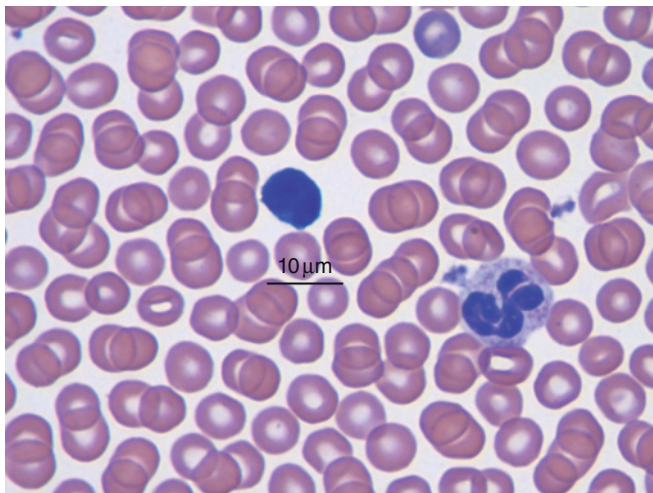


FIGURE 114.1 Erythrocytes with moderate anisocytosis mild polychromasia, several platelets, and a neutrophil with a few small pale eosinophilic cytoplasmic granules from a Syrian hamster; Wright's stain; 100× objective.

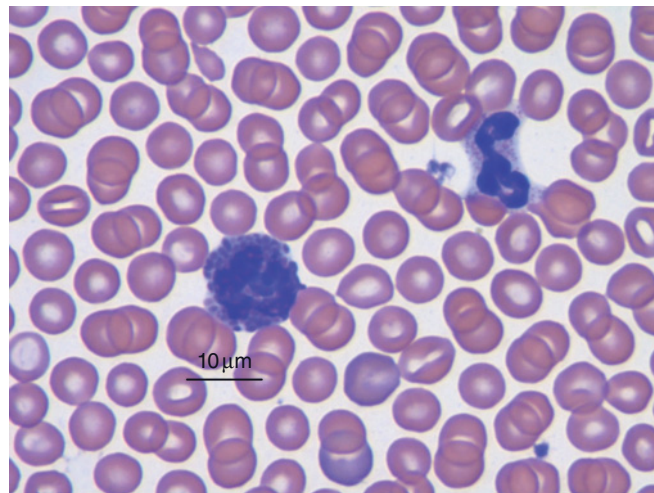


FIGURE 114.2 Neutrophil with a basophil from a Syrian hamster; Wright's stain; 100× objective.

Leukocytes

Neutrophils, also referred to as heterophils, have annular lobulated condensed nuclei and contain round- or rod-shaped acidophilic cytoplasmic granules. (Figs. 114.1 and 114.2).¹⁸ Neutrophils are either segmented or unsegmented and generally have a diameter between 10 and 12 μm . Two classes of lymphocytes, small and large (with the small form in greater prevalence), comprise between 60% and 80% of all circulating white blood cells (WBCs; Figs. 114.3 and 114.4).¹⁸ The nucleus of the hamster lymphocyte is round, condensed to the point that it almost seems granulated, and is infrequently indented.²⁰ A narrow band of blue-staining cytoplasm surrounds the lymphocyte nucleus and may contain azure granules. Occasionally, the band of cytoplasm of some lymphocytes may be reduced to the

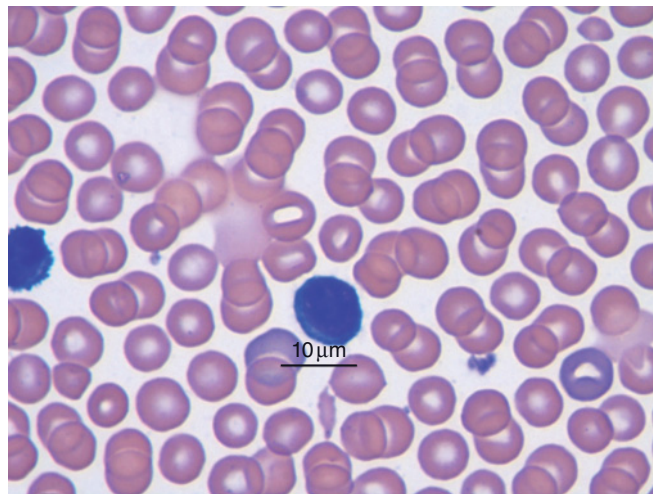


FIGURE 114.3 Small lymphocytes from a Syrian hamster; Wright's stain; 100× objective.

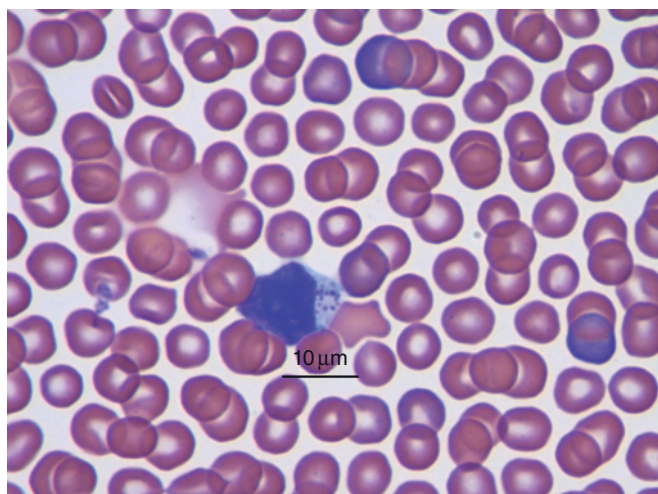


FIGURE 114.4 Large lymphocyte with a few small cytoplasmic dark basophilic granules from a Syrian hamster; Wright's stain; 100× objective.

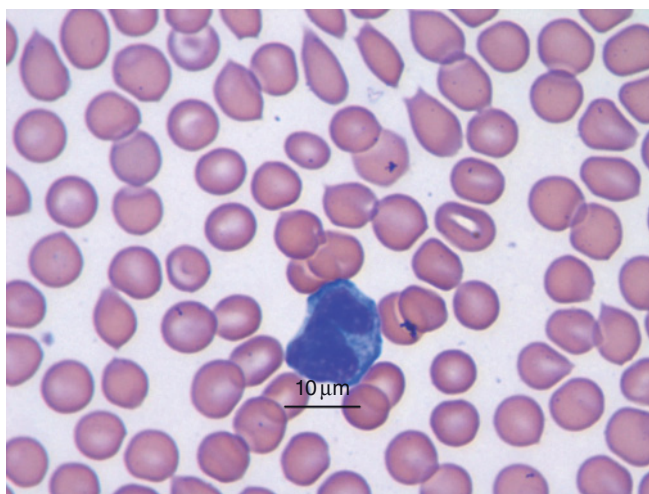


FIGURE 114.6 Monocyte from a Syrian hamster; Wright's stain; 100× objective.

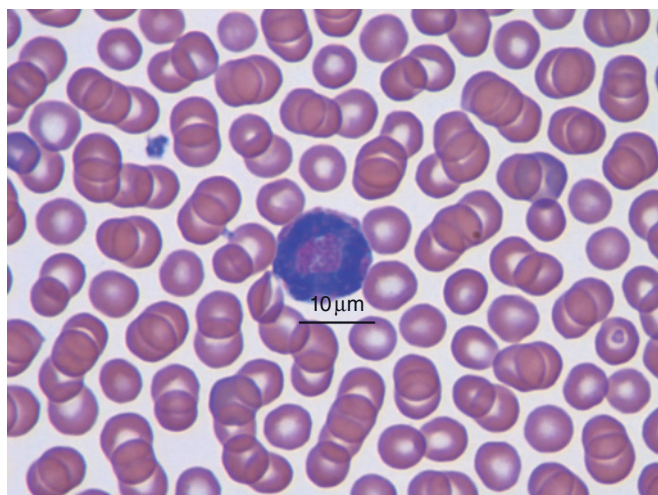


FIGURE 114.5 Eosinophil from a Syrian hamster; Wright's stain; 100× objective.

extent that the cells may be mistaken as basophils, although basophils are rarely observed (Fig. 114.2). The morphology of eosinophils, basophils, and monocytes is similar to that of other rodents. Eosinophils are relatively rare and may not be observed in some animals. Eosinophils have an annular nucleus, sometimes slightly twisted, that fills the periphery of the cell as a wide condensed band, surrounded by a narrow zone of cytoplasm (Fig. 114.5).²⁰ The closely packed cytoplasmic granules in the eosinophils of hamsters are shaped like short rods, in contrast to the more rounded granules observed in rats and mice. Monocytes are the largest cells observed in hamster blood and are characterized as having a large, swollen, indented, or trilobulate nucleus and a delicately reticulated gray-blue cytoplasm (Fig. 114.6).²⁰

A diurnal variation in the numbers and types of leukocytes can be observed in the hamster. Leukocyte levels increase to a range of $8 \times 10^3/\mu\text{L}$ to $10 \times 10^3/\mu\text{L}$ of blood at night when the nocturnal animal is most active, peaking in the early morning around 12×10^3 leukocytes/ μL , with an increase of neutrophils rather than lymphocytes.²⁰ Because most clinical and research manipulations of hamsters occur during daylight hours when the animals would typically be asleep, the implications of diurnal variation on hematologic indices should be considered when interpreting results.

White blood cell counts decline during hibernation to approximately 2,500 cells/ μL in Syrian hamsters and to approximately 1,000 cells/ μL in European hamsters, with an observed neutrophil to lymphocyte ratio of 1.^{16,19} After awakening from hibernation, a pronounced leukocytosis occurs (i.e. range of 10,000–20,000 leukocytes/ μL , mean of 13,600 cells/ μL) with a predominant neutrophilia, totaling 70–90% of the cells counted.²⁰

BONE MARROW CYTOLOGY

The cellular constituents of the bone marrow of hamsters are quite similar to those observed in rats and mice. Reports differ widely as to the myeloid to erythroid (M:E) ratio in normal bone marrow, with Desai reporting the M:E ratio as 8–10:1, and Trincao reporting a much lower ratio of 1.7:1.^{5,24} The results of the latter study are presented in Table 114.4.

Myeloblasts are frequently observed in the bone marrow of hamsters and have a large swollen nucleus that may contain one or two nucleoli, and are surrounded by a narrow basophilic band of cytoplasm. Myelocytes, each with a large swollen nucleus, have a brighter appearing cytoplasm that may occasionally

TABLE 114.4 Bone Marrow Constituents in Hamsters^a

	Percentage
Erythrocytic Series	
Rubriblast	0.14
Prorubricyte	1.94
Basophilic rubricyte	9.34
Polychromatic rubricyte	22.1
Metarubricyte	2.39
<i>Total erythroid cells</i>	36.18
Other cells	
Lymphocyte	0.04
Monocyte	0.07
Plasma cell	0.47
RE cell	1.91
Other	0.03
Granulocytic Series	
Myeloblast	1.22
Promyelocyte	3.03
Myelocyte (neutrophil)	13.72
Myelocyte (eosinophil)	0.29
Metamyelocyte (neutrophil)	29.59
Metamyelocyte (eosinophil)	0.55
Metamyelocyte (basophil)	0.05
Neutrophil	12.69
Eosinophil	0.2
Basophil	0.1
<i>Total granulocytic cells</i>	61.41
Myeloid:erythroid ratio = 1.696:1.0	

^aData from reference 24.

contain a few violet granules. Giant cells may be numerous, plasma cells are occasionally observed, and distinct lymphocytes or monocytes are rarely seen.^{12,20}

OTHER BLOOD FINDINGS

Nucleated cell types other than WBCs are occasionally observed in hamsters. Intravascular fetal trophoblasts may be seen in blood of pregnant and post-partum hamsters and are considered incidental findings.³ Megakaryocytes have been reported in blood in association with extramedullary hematopoiesis.²³

OTHER HEMATOLOGIC VALUES

Reported coagulation values for Syrian hamsters are listed in Table 114.5. The sedimentation rate (mm/hr) for normal hamster blood was reported by a number of authors: 0.5 mm at 60 minutes, and 0.7 mm at 120 minutes;²⁰ 0.3–0.96 mm/hr,¹⁶ and 0.32–0.96 mm/hr (mean, 0.64 ± 0.16 mm/hr) for males, and 0.30–0.70 mm/hr (mean, 0.50 ± 0.10 mm/hr) for females.¹⁶ However, seasonal temperature variations may influence these

TABLE 114.5 Observed Coagulation Values in Hamsters

Parameter	Mean ± SD	Reference
Bleeding time (seconds)	109 ± 19	5
Clotting time (seconds)	143 ± 50	5
	60	20
Prothrombin time (seconds)	180 ± 42	13
	10.5 ± 0.2	5
Males	9.0 ± 0.8	2
	14.8 ± 1.0	7
	9.9 ± 1.2	4
Females	9.3 ± 1.8	4
	22.2 ± 2.1	13
Partial thromboplastin time (seconds)	24.4 ± 2.7	7

results as hamsters exposed to lower temperatures may have reduced coagulability.⁶

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Hematology of Camelids

SUSAN J. TORNQUIST

Erythrocytes

- Erythrocyte Parameters
- Erythrocyte Shape and Physical Properties
- Oxygen Diffusion and Oxygen-Hemoglobin Dissociation Curve
- Response to Anemia

Leukocytes

Platelets

Reference Intervals

- Age
- Gender
- Environment
- Stress
- Bone Marrow
- Sites
- Morphology
- Cell Distribution

Acronyms and Abbreviations

EDTA, ethylenediaminetetraacetic acid; Hgb, hemoglobin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume, M:E ratio, myeloid to erythroid ratio; PCV, packed cell volume; RBC, red blood cell; WBC, white blood cell.

The biological family Camelidae includes two Old World species, the Bactrian and dromedary camels and four New World species, the domestic llama and alpaca and the wild guanaco and vicuna. They are in the order Artiodactyla that also includes pigs, hippopotami, and ruminants including cattle, goats, and antelopes, among others. Fossil records indicate that Camelidae originated in North America with subsequent migration to Asia and Africa by the Old World camelids and to South America by the New World camelids.^{14,33} In these environments, the camels adapted to severe dry deserts and the South American camelids adapted to the high altitude altiplano.

Genetic relationships between the four South American camelid species are complex. Genetic analyses provide conflicting evidence that alpacas and llamas are descended from the guanaco and that alpacas are descended from vicunas, but have undergone extensive hybridization.^{14,31} In veterinary medicine, llamas and alpacas are often considered to be the same with respect to laboratory reference intervals and interpretation, but there are metabolic differences between these species and separate reference intervals should be used.³

Unique features of the Camelidae include their feet that are two-toed with footpads rather than hooves and their red blood cells (RBCs) that are elliptical in shape, unlike those of any other mammals.^{2,5,7,13}

ERYTHROCYTES

Erythrocyte Parameters

Healthy camelids have packed cell volumes (PCVs) that are usually slightly lower than those of other herbivores (Tables 115.1 and 115.2). They have higher RBC numbers but smaller RBC size. Red blood cell numbers in camelids are usually reported in the range $12 \times 10^6/\mu\text{L}$ to $14 \times 10^6/\mu\text{L}$ for South American camelids and some Asian camels^{2,7,12,13,37,40}, with lower numbers reported in some camel samples.^{22,23} The average RBC size in all camelid species is smaller than most other domestic species with mean corpuscular volumes (MCVs) reported in the range 21–28 fL (Tables 115.1 and 115.2).⁶

Hemoglobin (Hgb) concentrations in South American camelid RBCs are similar to those in horses, but higher than cattle.⁶ The Old World camelids have somewhat lower mean Hgb concentrations than the South American camelids (Tables 115.1 and 115.2). The mean corpuscular hemoglobin concentration (MCHC) of camelids is generally higher than that of horses, cows, and sheep. However, the mean corpuscular hemoglobin (MCH), which is an index of the average amount of Hgb per RBC, is usually lower than reported in species that have larger erythrocytes.⁶

Erythrocyte Shape and Physical Properties

The elliptical erythrocytes of camelids are flat and thin with no central pallor. Because of their shape, they are sometimes folded on blood smears.³⁷ Hemoglobin crystals that appear as darkly eosinophilic, dense, rhomboid-shaped structures, are frequently seen on blood smears from clinically-healthy, non-anemic camelids. The cause and significance of these structures is not known (Fig. 115.1).³⁷ Cabot rings are also occasionally seen in erythrocytes from clinically-healthy camelids. These are narrow, thread-like structures in the form of rings or figure eights within RBCs. They are thought to be denatured membrane proteins, and have no known clinical significance.¹³

There are several structural and physiologic features of camelid RBCs that contribute to their high resistance to osmotic lysis, decreased deformability, and high affinity for oxygen. These attributes are adaptive for life in areas of low water availability or lowered oxygen availability. Membranes of camelid RBCs contain higher concentrations of the integral membrane protein, band 3, compared to other species.¹⁶ In addition, increased cross-linking of band 3 with other membrane proteins in camelid erythrocytes probably contributes to their decreased deformability and resistance to hypotonic lysis.^{20,27} Although decreased deformability of RBCs could be disadvantageous for animals facing a variety of environmental pressures, the disadvantage is minimized by the shape and small size of camelid RBCs that allows them to easily pass through capillaries without having to deform.

Oxygen Diffusion and Oxygen-Hemoglobin Dissociation Curve

The size and shape of camelid RBCs facilitates the diffusion of oxygen by providing a higher effective surface area for gas exchange.³⁹ Goat RBCs that are of similar

size, but with a smaller surface area, have a much lower oxygen capacity as well as a lesser oxygen affinity for blood.³⁹

Another adaptation to life at high altitude and low oxygen tension is an oxygen-hemoglobin dissociation curve that is shifted to the left, especially at lower partial pressures of P_{O_2} , facilitating uptake of oxygen by the lung.³⁰ In addition, the percentage of alkali-resistant (fetal) Hgb with increased oxygen affinity is higher in adult alpacas than in adults of other species.³⁰

Response to Anemia

Anisocytosis, polychromasia, reticulocytes, and nucleated RBCs may be seen in response to anemia, but the response is not as predictable as it is in other species. In normal, non-anemic llamas, reticulocyte numbers are reported in the range 12,000–79,000/ μL of blood, (i.e. 0.2–0.6% or 0–0.4%).^{2,7,37} Mean reticulocyte percentages for normal alpacas and vicunas are reported to be 1.4% and 1.2%, respectively.²⁹ As many as 2–3 nucleated RBCs per 100 white blood cells (WBCs) may be seen in non-anemic llamas.²⁷ In alpacas made acutely and severely anemic by phlebotomy, reticulocyte percentages increased, but only to a maximum of 1.5%, with the peak reticulocyte response occurring at a mean of 10.4 days after induction of anemia.³⁶

Other morphologic features of camelid RBCs that are occasionally observed in anemia include dacryocytes, or tear-drop-shaped cells, spindle-shaped erythrocytes, and uneven distribution of Hgb in the cells (Fig. 115.2).^{24,25,31} Although these have been reported in association with iron deficiency anemia, they are seen in moderate to marked anemias of other etiologies (S.J. Tornquist, unpublished observations). Heinz bodies have been reported in two alpacas that had ingested red maple leaves.⁴ '*Candidatus* Mycoplasma haemolamae', a hemotropic mycoplasma, may be observed as small, round or ring-shaped basophilic organisms on the

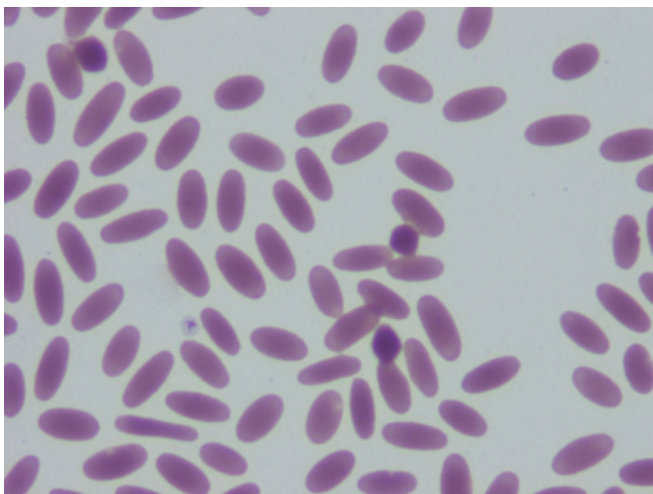


FIGURE 115.1 Hemoglobin crystals in alpaca blood.

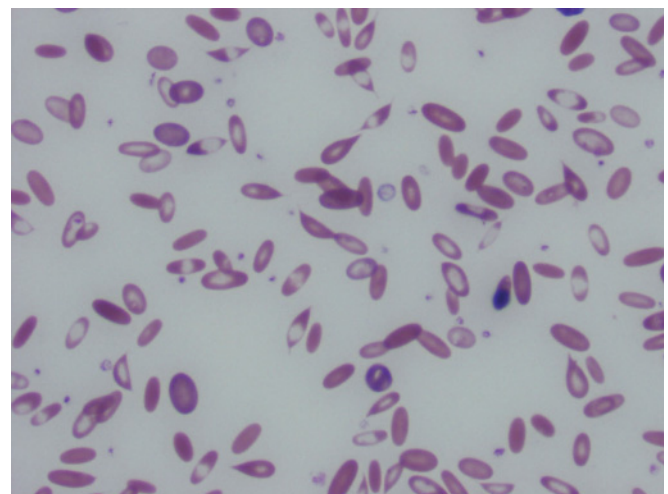


FIGURE 115.2 Erythrocyte dyscrasia in anemic alpaca.

TABLE 115.1 Erythrocyte Parameters of South American Camelids

Reference	Species	No.	Gender/Age	RBC ($\times 10^6/\mu\text{L}$)		PCV (%)		Hgb (g/dL)		MCV (fL)		MCH (pg)		MCHC (g/dL)		Reticulocytes (%)		Reticulocytes ($\times 10^9/\mu\text{L}$)
				Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Range
7	Llama	20	NS (<1 mo)		9.6–15.2		24–35		10.1–14.9		22.2–26.1		9.0–11.1		39.4–44.1			0–7.5
7	Llama	30	NS (2–6 mo)		11.4–17.2		28–42.5		12.7–18.1		21.5–29.0		9.4–11.9		39.7–44.9			0–2.1
7	Llama	35	NS (6–18 mo)		10.0–16.1		25.5–38.5		11.1–16.7		22.4–28.0		9.5–11.9		39.3–45.5			0–0.4
7	Llama	35	M (adult)		10.5–17.1		27–45		11.7–19.1		22.5–29.1		10.3–12.5		39.9–48.7			0–0.5
7	Llama	54	F (adult)		10.6–17.2		27.5–45		12.5–19.2		22.9–30.2		10.0–12.7		40.0–46.7			0–0.3
37,38	Llama	38	NS (adult)	14.1 \pm 1.4	11.3–17.7		29–39	15.2 \pm 1.3	12.8–17.7	23.8 \pm 1.9	20.9–28			44.9 \pm 0.8	43.1–46.6			12–79
2	Llama	20	NS (adult)	12.3 \pm 1.5	10.3–15.0	31.4 \pm 3.1	28–35	13.7 \pm 1.4	12.0–17.5	25.5 \pm 2.8	21.1–31.0	11.1 \pm 1.1	9.6–13.6	43.6 \pm 2.8	38.6–48.0	0.4 \pm 0.1		
13	Llama	14	NS (adult)	11.4 \pm 2.9	9.6–14.6	30 \pm 5	27–36	13.7 \pm 2.4	10.3–17.0	26.9 \pm 1.3	25.0–28.7	12.0 \pm 0.6	10.9–12.9	44.8 \pm 2.3	42.9–46.6	0.2 \pm 0.2		
13	Llama	10	NS (juvenile)	11.9 \pm 0.8	10.9–13.6	31 \pm 3	27–35	13.5 \pm 0.9	12.6–15.4	25.8 \pm 1.8	23.6–29.9	11.4 \pm 0.5	10.6–12.2	44.5 \pm 2.4	40.6–47.0	0.4 \pm 0.6		
5	Alpaca	40 ^b	NS			36.0 \pm 1.5	33–42											
^a	Alpaca	37	NS (adult)	13.72 \pm 1.43	9.51–17.02	29 \pm 2.5	25–37	13.7 \pm 1.1	11.5–16.4	21.9 \pm 2.1	18.5–28.4	10.0 \pm 1.0	8.5–13.9	45.9 \pm 1.5	41.9–48.9			
12	Alpaca	29	NS (10–18 mo)		11.5–14.1	39.1 \pm 5.1	30–41		13.1–18.5		23–32				38.2–55.7			
15	Guanaco	20	NS				30–48											
13	Guanaco	17	NS (adult)	13.1 \pm 1.3	11.3–15.2	36 \pm 3.0	28–42	15.7 \pm 1.7	12.3–18.9	27.5 \pm 1.7	24.9–30.8	12.1 \pm 0.8	10.5–13.7	43.8 \pm 0.6	40.0–47.4	0.9 \pm 1.0		
13	Guanaco	5	NS (juvenile)	14.9 \pm 0.4	13.5–14.6	36 \pm 3	34–47	15.9 \pm 0.7	14.9–16.5	25.6 \pm 1.7	24.9–30.8	11.2 \pm 0.7	10.2–11.8	43.9 \pm 0.6	43.2–44.6	0.6 \pm 0.3		
29	Vicuna ^b	12	Male (adult)	14.4 \pm 0.4		36 \pm 0.9		13.5 \pm 0.5		27.4 \pm 0.6		10.2 \pm 0.3		37.5 \pm 0.5				

^aFrom Oregon State University Clinical Pathology Laboratory.

^bAt 4200 m elevation.

NS, not specified.

TABLE 115.2 Erythrocyte Parameters of Camels

Reference	Species	No.	Gender/Age	RBC ($\times 10^6/\mu\text{L}$)		PCV (%)		Hgb (g/dL)		MCV (fL)		MCH (pg)		MCHC (g/dL)	
				Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range
11	Dromedary	13	NS (adult)	5.14 \pm 0.27	3.5–6.1	21.5 \pm 1.1	15.0–27.0	9.26 \pm 0.47	7.0–11.0	40.41 \pm 4.1	17.4–62.6	18.1 \pm 1.1	13.4–28.0	42.5 \pm 2.1	29.6–64.7
22	Dromedary (racing)	100	NS (adult)	7.97 \pm 0.99	7.00–10.55	33 \pm 5	16–42	13.98 \pm 1.35	11.0–16.0	42.8 \pm 4.7	14.0–48.4	17.8 \pm 2.0	11.8–20.8	42.3 \pm 5.9	33.0–61.7
40	Bactrian	25	M (adult)	12.34 \pm 2.41	6.23–15.25	34.4 \pm 2.6		10.96 \pm 2.40		25.4 \pm 5.5		8.1 \pm 1.9			
40	Bactrian	25	F (adult)	11.52 \pm 3.07		30.2 \pm 3.4		9.91 \pm 1.96		25.4 \pm 5.1		8.7 \pm 1.9		31.8 \pm 3.2	
40	Bactrian	50	NS (adult)	12.56 \pm 3.60	6.23–15.25	31.2 \pm 3.1	21.3–42.5	10.46 \pm 2.42	7.64–13.45	25.3 \pm 5.1	19.7–34.8	8.3	7.0–10.2	32.9 \pm 2.9	22.4–42.9

NS, not specified.

margin of the erythrocytes, or in the background, if blood smears are not made promptly after blood collection.^{19,28,35}

LEUKOCYTES

Leukocytes of camelids do not have the unique features of their RBCs. In general, the white blood cell (WBC) count for camelids is somewhat higher than for many similar species. Reference intervals are in the range 8,000–22,000/ μL (Tables 115.3 and 115.4). In contrast, typical reference intervals for total leukocyte numbers of cattle and sheep are 4,000–12,000/ μL and for horses 5,500–12,500/ μL .⁶

The neutrophil to lymphocyte ratio in adult llamas and camels is about 1.5 which is more similar to humans, dogs, cats, and horses than it is to ruminants (Tables 115.3 and 115.4).^{7,9,37} The neutrophil to lymphocyte ratio was reported to be lower (0.5–2.9) in a group of 29 alpacas, but these were 10–18 months old so the difference may be more age- than species-based.¹² Band neutrophils are seen in blood in response to inflammation and their morphology is like that of other species.¹⁰

Eosinophil numbers tend to be higher in healthy adult llamas than in other species.^{7,13} Eosinophils are usually hyposegmented with band-shaped to bilobed nuclei (Fig. 115.3).³⁷

Camelid lymphocytes vary in size as they do in ruminants. The percentage of granular lymphocytes in circulation in healthy llamas has been reported to be higher than that seen in cattle and horses. In three different studies, the percentage of granular lymphocytes was 7–29%, 1–19%, and 10–30% of blood lymphocytes.^{2,9,37} Granules vary in size (Fig. 115.4).²

PLATELETS

Platelets from camelids are generally small in volume, similar to those from cows and horses, round to irregular, and contain azurophilic granules (Table 115.3).^{2,8,17,34} Gader et al. recently showed that camel platelets, like those from cattle and horses, lack the open canalicular system that is present in platelets from other species.⁸

REFERENCE INTERVALS

There are challenges in comparing published reference intervals between camelids of different ages, sexes, species, and physiologic conditions, because so many of the reference intervals are based on relatively small numbers of animals and because sampling conditions can vary greatly.

Age

Some of the more frequently reported age-related differences in camelids include lower PCV and Hgb, and

higher absolute lymphocyte and platelet numbers in juvenile llamas and guanaco compared to adults.^{7,13,21} Juvenile llamas have a lower neutrophil to lymphocyte ratio and juvenile alpacas have fewer absolute eosinophil numbers compared to adults.⁷

Gender

There are few reported significant gender differences in camelid reference intervals. Packed cell volume is reported to be higher in adult male llamas than in adult female llamas.²¹ In studies of adult Bactrian and dromedary camels, no significant differences in hematologic parameters were reported between males and females.^{22,40} Eosinophil numbers were reported to be significantly higher in females than males in a group of 29 10–18 month old alpacas.¹²

Environment

In studies of captive ($n = 17$) versus free-ranging guanaco ($n = 20$), the captive population had a lower PCV range (28–42%) than did the free-ranging population (30–48%). In addition, the range of the total leukocyte count was much higher and wider ($14.3 \times 10^6/\mu\text{L}$ to $53.4 \times 10^3/\mu\text{L}$) in the free-ranging population than in the captive guanacos ($1.5 \times 10^6/\mu\text{L}$ to $9.2 \times 10^3/\mu\text{L}$). Animals in both populations were reported to be clinically healthy, but these differences may reflect response to handling and stress.^{13,15}

The effects of altitude on hematologic parameters in camelids are not clear. For example, Ellis reported a PCV range of 33–42% for 40 alpacas at the La Raya, Peru, experimental station located at an altitude of 4,200 meters, but Reynafarje et al reported a range of 34–37% for 12 alpacas at the same station.^{5,29} Fowler reported a range of PCVs for 116 alpacas at 3,900 meters as 24–45%.⁶ The PCV range for 37 healthy Oregon alpacas living at an altitude of approximately 68 meters was 25–37% and for 29 alpacas in Australia (altitude not reported) was 30–42%.¹²

Stress

The effects of stress and excitement on hematologic parameters in camelids is also somewhat unclear. Miller and Banchemo showed that llamas given injections of epinephrine to simulate stress or excitement had increases in PCV of approximately 10% at 3 and 6 minutes post-injection. The PCV typically dropped to resting level in 20 minutes.²¹ These findings suggest a large splenic reserve with splenic contraction releasing RBCs into the circulation in response to excitement or stress in the llama. In contrast, the change in PCV in racing camels is small in response to the stress of racing, suggesting that there is not a large splenic reservoir of RBCs released.³²

Physiologic neutrophilia, a shift of marginated neutrophils to the circulating pool in response to catecholamine release during handling and blood sampling,

TABLE 115.3 Leukocyte and Platelet Parameters of South American Camelids

Reference	Species	No.	Gender/Age	WBC ($\times 10^3/\mu\text{L}$)		Band Neutrophils		Neutrophils		Lymphocytes		Monocytes		Eosinophils		Basophils		Platelets ($\times 10^3/\mu\text{L}$)		
				Mean \pm SD	Range	Mean \pm SD (%)	Range ($\times 10^3/\mu\text{L}$)	Mean \pm SD (%)	Range ($\times 10^3/\mu\text{L}$)	Mean \pm SD (%)	Range ($\times 10^3/\mu\text{L}$)	Mean \pm SD (%)	Range ($\times 10^3/\mu\text{L}$)	Mean \pm SD (%)	Range ($\times 10^3/\mu\text{L}$)	Mean \pm SD (%)	Range ($\times 10^3/\mu\text{L}$)	Mean \pm SD	Range	
7	Llama	20	NS (<1 mo)		7.1–19.4		0–0.487		1.13–14.56		1.70–4.73		0–1.42		0–1.1		0–0.15			
7	Llama	30	NS (2–6 mo)		9.1–22.9		0–0.099		4.30–14.27		3.05–10.67		0.15–1.39		0.08–2.82		0–0.23			
7	Llama	35	NS (6–18 mo)		10.2–23.6		0–0.090		4.22–12.95		1.88–7.68		0–1.41		0.30–6.94		0–0.39			
7	Llama	35	M (adult)		7.9–23.6		0–0.021		4.62–16.16		0.98–4.92		0–0.94		0.79–4.21		0–0.34			
7	Llama	54	F (adult)		8.3–19.2		0–0.145		5.11–14.15		0.65–4.74		0.11–1.00		0.61–5.51		0–0.30			
37	Llama	38	NS (adult)		7.5–21.5				4.6–16.0		1.0–7.5		0.05–0.8		0–3.30		0–0.40			
2	Llama	20	NS (adult)	15.6 \pm 4.0	9.7–22.0		0–0.35		6.75–14.75		1.36–5.75		0–0.690		0.88–4.00		0–0.11	410 \pm 170	210–800	
13	Llama	14	NS (adult)	8.7 \pm 1.4	6.2–10.0				3.2–7.7		0.6–4.1		0–0.7		0–1.6		0–0.1	332	180–441	
13	Llama	10	NS (juvenile)	12.4 \pm 3.7	5.6–16.9				3.2–7.7		2.0–11.2		0–0.7		0–1.7		0–1.0	500 \pm 229	268–912	
5	Alpaca	40 ^b	NS	11.0 \pm 1.0	9.0–12.0			36.5 \pm 11.3		49.9 \pm 11.9		1.2 \pm 1.5		11.9 \pm 7.4		1.0 \pm 1.2				
^a	Alpaca	37	NS (adult)	16.97 \pm 3.23	10.5–25.3	1.15 \pm 0.38	0–0.36	49.73 \pm 11.87	2.98–15.26	34.16 \pm 10.40	2.81–10.14	2.27 \pm 0.21	0–1.31	12.24 \pm 5.69	0.26–6.38	1.67 \pm 1.09	0–0.71	286 \pm 111	98–546	
12	Alpaca	29	NS (10–18 mo)		6.4–21.6	0–2 (range)	0–0.2	42–75 (range)	3.4–16.2	19–53 (range)	2.3–7.3	0–4 (range)	0–0.8	2–20 (range)	0.2–2.7					
15	Guanaco	20	NS	30.14 \pm 10.85	14.3–53.4			48.45 \pm 10.08		44.8 \pm 9.99		2.8 \pm 2.31		3.85 \pm 2.91		0.1 \pm 0.45				
13	Guanaco	17	NS (adult)	10.4 \pm 3.2	2.7–14.7				1.5–9.2		0.9–5.5		0–0.7		0–2.1		0	282 \pm 108	148–435	
13	Guanaco	5	NS (juvenile)	13.3 \pm 3.4	9.9–16.6				5.9–8.0		3.0–8.9		0–0.1		0.4–1.1		0	419 \pm 43	358–454	
29	Vicuna ^b	12	M (adult)	12.2 \pm 0.81				46.8 \pm 3.1		33.8 \pm 3.0		2.4 \pm 0.3		14.6 \pm 2.2						

^aFrom Oregon State University Clinical Pathology Laboratory.

^bAt 4200 m elevation.

NS, not specified.

TABLE 115.4 Leukocyte Parameters of Camels

Reference	Species	No.	Gender/Age	WBC ($\times 10^3/\mu\text{L}$)		Neutrophils		Lymphocytes		Monocytes		Eosinophils		Basophils	
				Mean	Range	Mean (%)	Range ($\times 10^3/\mu\text{L}$)	Mean (%)	Range ($\times 10^3/\mu\text{L}$)	Mean (%)	Range ($\times 10^3/\mu\text{L}$)	Mean (%)	Range ($\times 10^3/\mu\text{L}$)	Mean (%)	Range ($\times 10^3/\mu\text{L}$)
11	Dromedary	13	NS (adult)	10.42 \pm 0.56	6.50–12.85	55 \pm 4.61	2.39–2.47	28.76 \pm 4.83	1.72–10.15	3.69 \pm 0.61	0.09–0.58	2.61 \pm 0.61	0–0.80	0	0
22	Dromedary (racing)	100	NS (adult)	11.44 \pm 2.73	7.0–17.0										
40	Bactrian	25	M (adult)	6.99 \pm 2.34		57.22 \pm 8.21		32.73 \pm 7.23		0.58 \pm 0.68		8.53 \pm 1.22		0.55 \pm 0.76	
40	Bactrian	25	F (adult)	6.59 \pm 2.12		58.98 \pm 10.23		30.45 \pm 6.98		0.72 \pm 0.54		6.24 \pm 3.11		0.47 \pm 0.62	
40	Bactrian	50	NS (adult)	6.86 \pm 2.26	4.66–9.95	58.85 \pm 9.34		31.24 \pm 5.22		0.62 \pm 0.69		6.76 \pm 2.56		0.49 \pm 0.65	

NS, not specified.

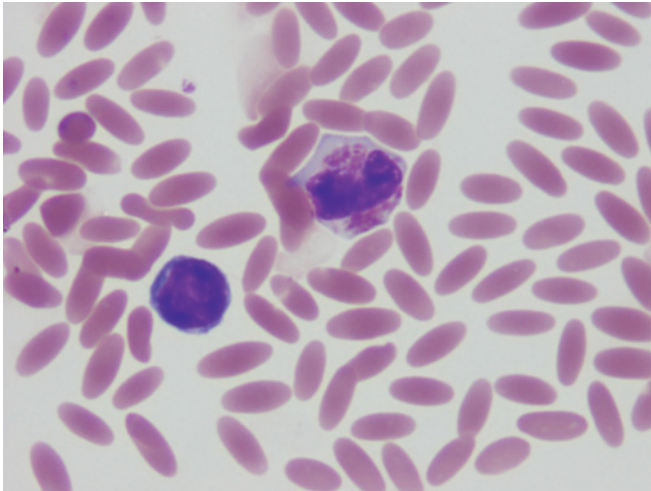


FIGURE 115.3 Lymphocyte and eosinophil in llama blood.

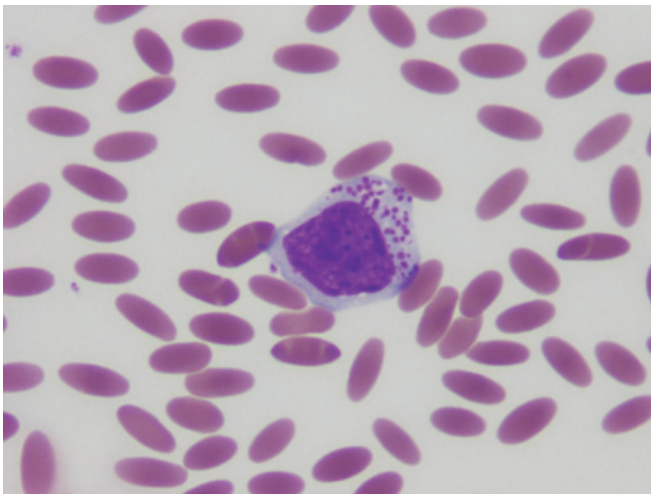


FIGURE 115.4 Large granular lymphocyte in llama blood.

may account for at least some of the discrepancies reported in neutrophil: lymphocyte ratios in camelid species. While the neutrophil: lymphocyte ratio is most frequently reported as being greater than unity and thus more similar to horses than to sheep and cattle, at least one author has reported a lower ratio and suggests that catecholamine-mediated neutrophilia affects the ratio in many studies.^{5,6}

BONE MARROW

Sites

Bone marrow is most frequently obtained from the sternum or ribs of camelids. Andreasen, et al. aspirated bone marrow from the sternum of llamas after sedation with intravenous xylazine. The llamas were sampled either while standing or in left lateral recumbency.¹ McKenzie et al. obtained sternal bone marrow samples

from alpacas also sedated with intravenous xylazine. These alpacas were placed in lateral recumbency and bone marrow was obtained from the midline of the sternum at the level of the caudal aspect of the elbow when positioned naturally.¹⁸ Bone marrow aspiration from non-sedated dromedary camels has been described by Nazifi et al. who took samples from the 5th-8th ribs.²⁶ In all cases, the site was aseptically prepared and an 11-16 gauge bone marrow biopsy needle used to obtain samples. Bone marrow samples are most frequently aspirated into EDTA but may be collected with no anticoagulant if bone marrow smears are prepared immediately. Slides are air-dried and stained with appropriate stains including Wright's or Wright-Giemsa for general evaluation or with Perl's iron stain to assess iron stores.

Morphology

Cellularity of normal camelid bone marrow is reported to be similar to or somewhat increased in comparison with other mammals. In studies of seven llamas and 20 dromedary camels cellularity of 50-85% was noted.^{1,26} The morphology and maturation of erythroid cells in bone marrow was similar to that seen in other species in both llamas and camels. Erythroid cells started to change from round to elliptical as they became less polychromatophilic.¹ No dacryocytes, spindle-shaped erythrocytes or uneven Hgb distribution were reported in bone marrow samples. No unique features of leukocyte morphology or maturation have been reported in camelid bone marrow samples. Percentages of bone marrow eosinophils are increased in comparison to other species, which parallels the higher percentages seen in blood.¹ Megakaryocyte numbers and morphology appear to be similar to those seen in other species. Stainable iron was present in moderate amounts in macrophages in the bone marrow of both llamas and dromedary camels.^{1,26}

Cell Distribution

The average M:E ratio in bone marrow of healthy camelids is similar to that of other species. It is reported to average 1.21 in dromedary camels,²⁶ 0.9-2.9 in llamas,^{1,6} and 0.47-1.01 in alpacas.¹⁸ Myeloid:erythroid ratios in bone marrows from small numbers of alpacas, llamas, and vicunas living at 4,200 meters (13,800 feet) were reported to be much lower, with an average of approximately 0.5.²⁹

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Hematology of Cervids

KATIE M. BOES

Properties of Blood Cells

Erythrocytes
Leukocytes
Platelets

Reference Intervals

Influence of Age
Influence of Gender
Influence of Reproductive Status
Influence of Stress and Handling
Response to Disease

Acronyms and Abbreviations

CBC, complete blood count; Hct, hematocrit; Hgb, hemoglobin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PCV, packed cell volume; PLT, platelet; RBC, red blood cell; TPP, total plasma protein; WBC, white blood cell.

The Cervidae family is composed of various species of deer, wapiti, elk, moose, and reindeer, also known as caribou, that have adapted to a variety of climactic and geographic areas throughout the world.^{9,11} Consequently, it is difficult to define the “normal” hematology for such a wide range of species and subspecies. The hematologic profile of cervids is influenced by a variety of factors: age, gender, reproductive status, seasonality, stress, method of capture, and disease all have an effect. Therefore it is useful to establish “normal” reference intervals for each animal population in accordance with its respective life stage and method of capture. Furthermore, the most useful data for an individual animal are to reference previous data that were taken from the same individual under the same conditions. With this said, this chapter will characterize the “normal” hematology of a variety of cervids. However, due to their differences in red blood cell (RBC) morphology, reindeer will be discussed in Chapter 119.

PROPERTIES OF BLOOD CELLS

Erythrocytes

Members of the family Cervidae have erythrocytes with unique characteristics. The erythrocytes circulate in the vasculature as round cells, which are slightly smaller

than bovine red cells. However, shortly after phlebotomy, deer red cells tend to assume a sickle shape (Fig. 116.1A, B). The cells are not sickled when first removed from the body, but the shape change occurs if the blood is alkalinized, oxygenated, or left to stand at room temperature or at 4°C.^{18,19} This sickling phenomenon was first reported in 1840 by Gulliver,¹⁰ and has since been observed in a variety of cervids, including barasingha (*Rucervus duvaucelii*), barking deer (*Muntiacus muntjak*), chital deer (*Axis axis*), fallow deer (*Dama dama*), hog deer (*Axis porcinus*), mule deer (*Odocoileus hemionus*), Père David's deer (*Elaphurus davidianus*), red deer (*Cervus elaphus*), Reeve's muntjac (*Muntiacus reevesi*), Rocky Mountain wapiti (*Cervus elaphus nelsoni*), rusa deer (*Rusa timorensis russa*), sambar deer (*Rusa unicolor*), sika deer (*Cervus nippon nippon*), and white-tailed deer (*Odocoileus virginianus*).^{2,7,9-12,23,33} The sickling effect can be prevented by acidifying the blood. At pH 7.0, few red cells are sickled; at pH 7.4, most of the red cells have a sickle shape.³³ Furthermore, sickling may be enhanced by oxygenation of the red cells.¹⁸ Subsequent addition of carbon dioxide will reverse the sickling, and round the cells.¹⁸ In vivo, transient alkalosis and increased oxygenation may have similar effects on red cell morphology.²⁰ There are at least two exceptions within the Cervidae family: the erythrocytes of reindeer (*Rangifer tarandus*) and Eurasian elk (*Alces alces*) do not assume the sickle shape when exposed to oxygen. Fig. 116.1C, D show parasitized non-sickled red cells in wapiti blood.³³

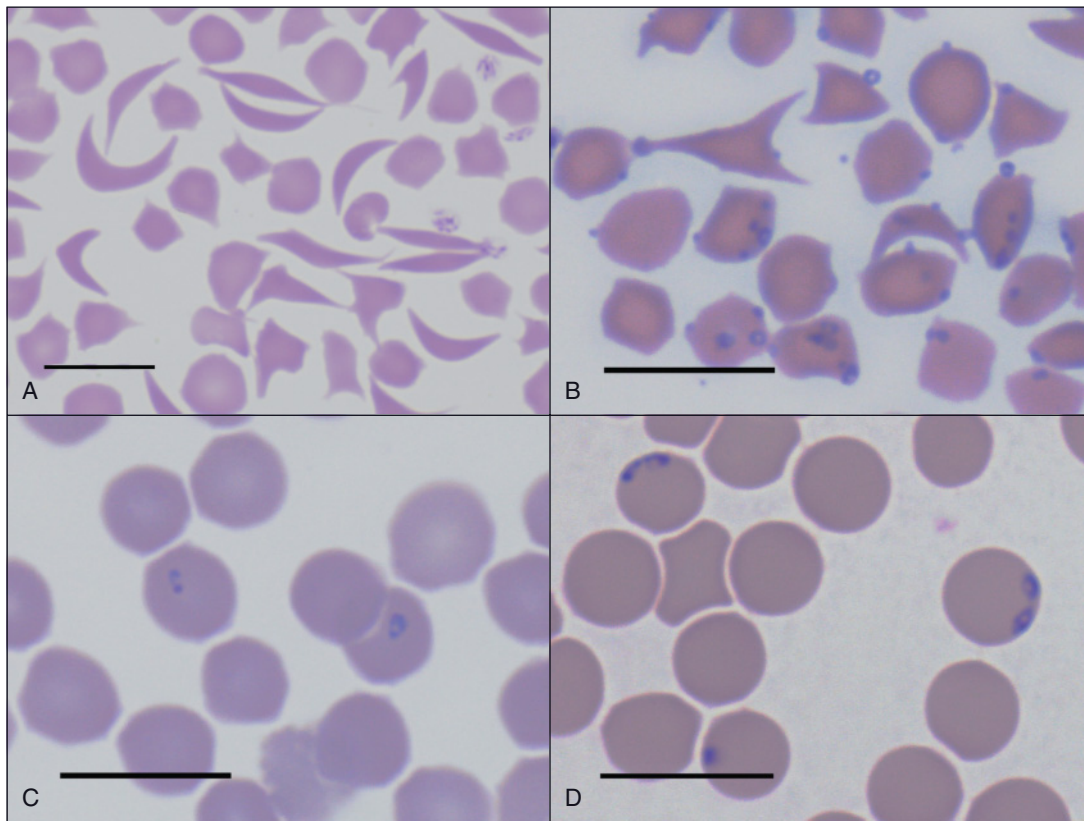


FIGURE 116.1 Normal and parasitized cervid erythrocytes. Modified Wright stain; bar = 10 μ m. (A) Deer, erythrocytes with normal sickled appearance. Platelets are small and moderately granulated. (B) Deer, erythrocytes with *Mycoplasma ovis*-like organisms. (C) Wapiti (elk), round, non-sickled erythrocytes containing centrally located small, unpaired *Theileria cervi* organisms. (D) Wapiti (elk), erythrocytes with peripherally located paired *Babesia odocoilei* organisms.

The sickling effect is due to the formation of insoluble tactoids of variant forms of hemoglobin (Hgb) in the oxygenated state.¹⁹ Deer erythrocytes contain several morphologic forms of Hgb, each of which has different electrophoretic properties and is associated with a distinct morphologic red cell shape.¹⁹ These various morphologic forms are composed of 21 structurally different hemoglobins.²⁰ Consequently, an individual deer may have 1–3 different morphologic and 1–6 different structural forms of Hgb.²⁰ Morphologic types I and III Hgb are present in approximately half the deer population.¹⁹ Hemoglobin type II alone or in combination with Hgb types I, III, or IVb is often associated with the development of a matchstick shape, which forms subsequent to the development of sickling. Red cells that contain Hgb type IVa may assume a burr shape. Other red cell shapes include the traditional crescent or holly leaf. The sickle effect does not occur if Hgb type V or VII is present.¹⁹ The sickling phenomenon is an interesting laboratory observation but does not appear to have a deleterious effect on the deer.¹⁹ One in vivo study of cervid erythrocytes demonstrated a RBC lifespan comparable with those of other domestic species.²³ The erythrocytes of five white-tailed deer, one sika deer,

and one Rocky Mountain wapiti survived in circulation for 125, 159, and 149 days, respectively.²³

As seen in other mammals, there is a relationship between body size, RBC size, and RBC count in cervids. Observations on several species of deer have revealed a direct relationship between RBC size and body size in members of the Cervidae. Furthermore, there is an indirect relationship between RBC size and RBC number.¹³ In white-tailed deer, discoid erythrocytes measure 3.5–4.5 μ m in diameter.¹⁹

Leukocytes

The most extensive characterization of cervid leukocyte morphology, cytochemical staining, and ultrastructural properties has been conducted on reindeer white blood cells (see Chapter 119). Deer and wapiti leukocytes show similar morphology using Romanowsky stains (Figs. 116.2–116.4). Of the cervid leukocytes, lymphocytes and neutrophils are the most numerous. Neutrophil to lymphocyte ratios are typically less than, or equal to unity, but some studies have shown that neutrophils predominate (Tables 116.1 and 116.2).

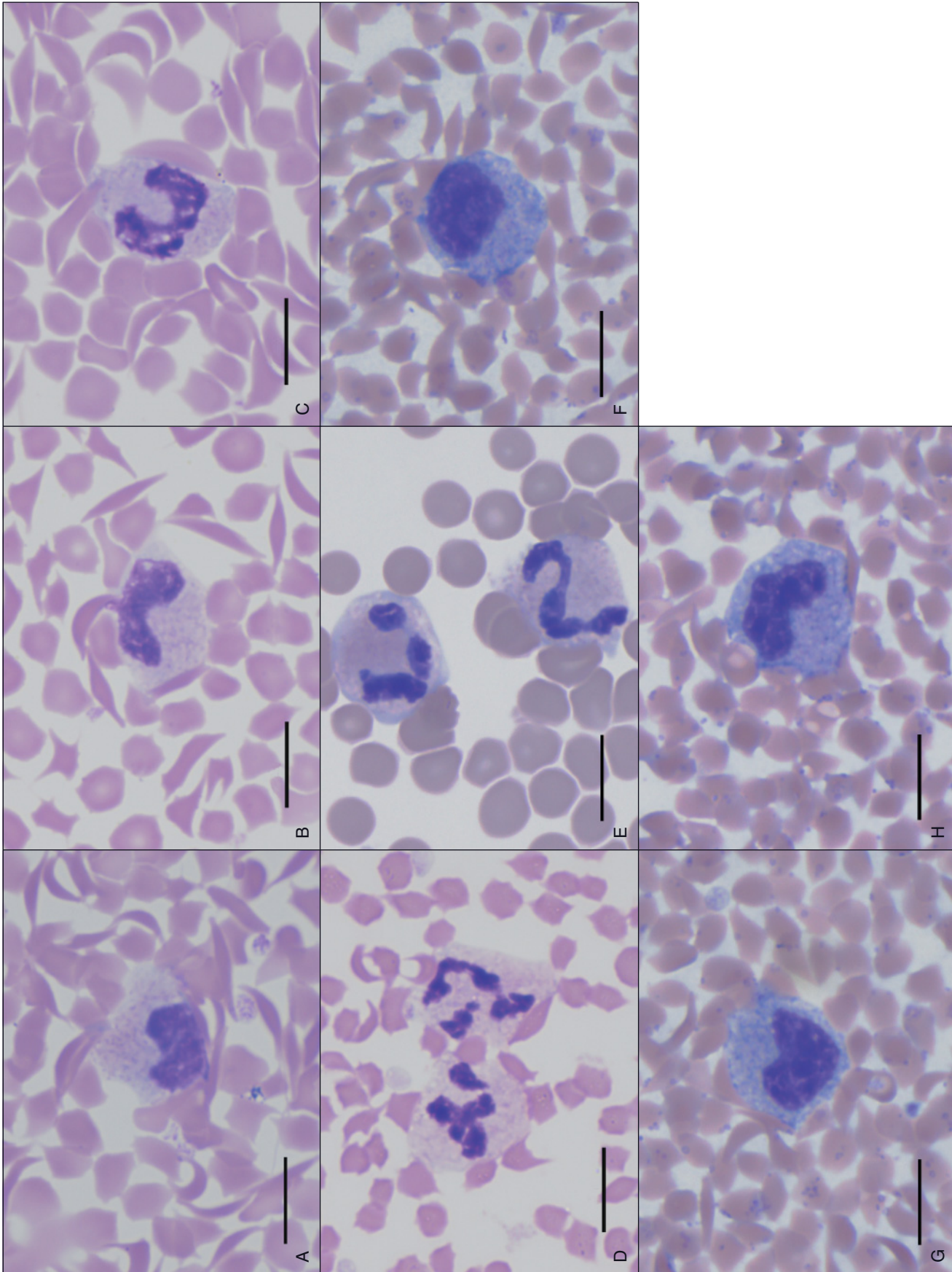


FIGURE 116.2 Normal and toxic cervid neutrophils. Modified Wright stain; bar = 10µm. (A) Deer, metamyelocyte neutrophil. (B) Deer, early band neutrophil. (C) Deer, late band neutrophil. (D) Deer, segmented neutrophils. (E) Wapiti (elk), segmented neutrophils. (F) Deer, toxic myelocyte neutrophil and *Mycoplasma* infection. (G) Deer, toxic metamyelocyte neutrophil and *Mycoplasma* infection. (H) Deer, toxic band neutrophil and *Mycoplasma* infection.

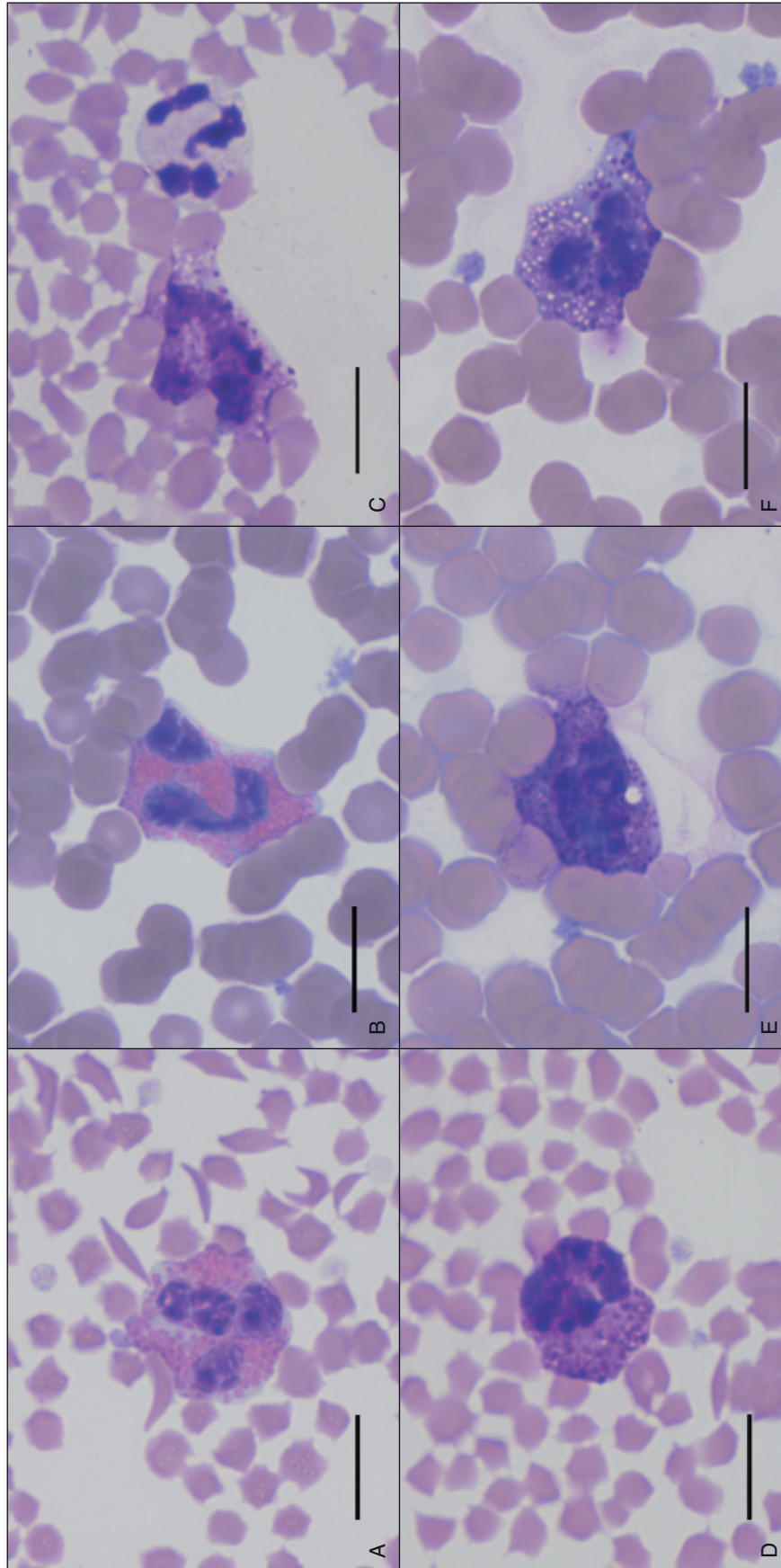


FIGURE 116.3 Normal circulating cervid eosinophils and basophils. Modified Wright stain; bar = 10 µm. (A) Deer, eosinophil. (B) Wapiti (elk), eosinophil. (C) Deer, basophil and segmented neutrophil. (D) Deer, basophil. (E) Wapiti (elk), highly granulated basophil. (F) Wapiti (elk), partially vacuolated basophil.

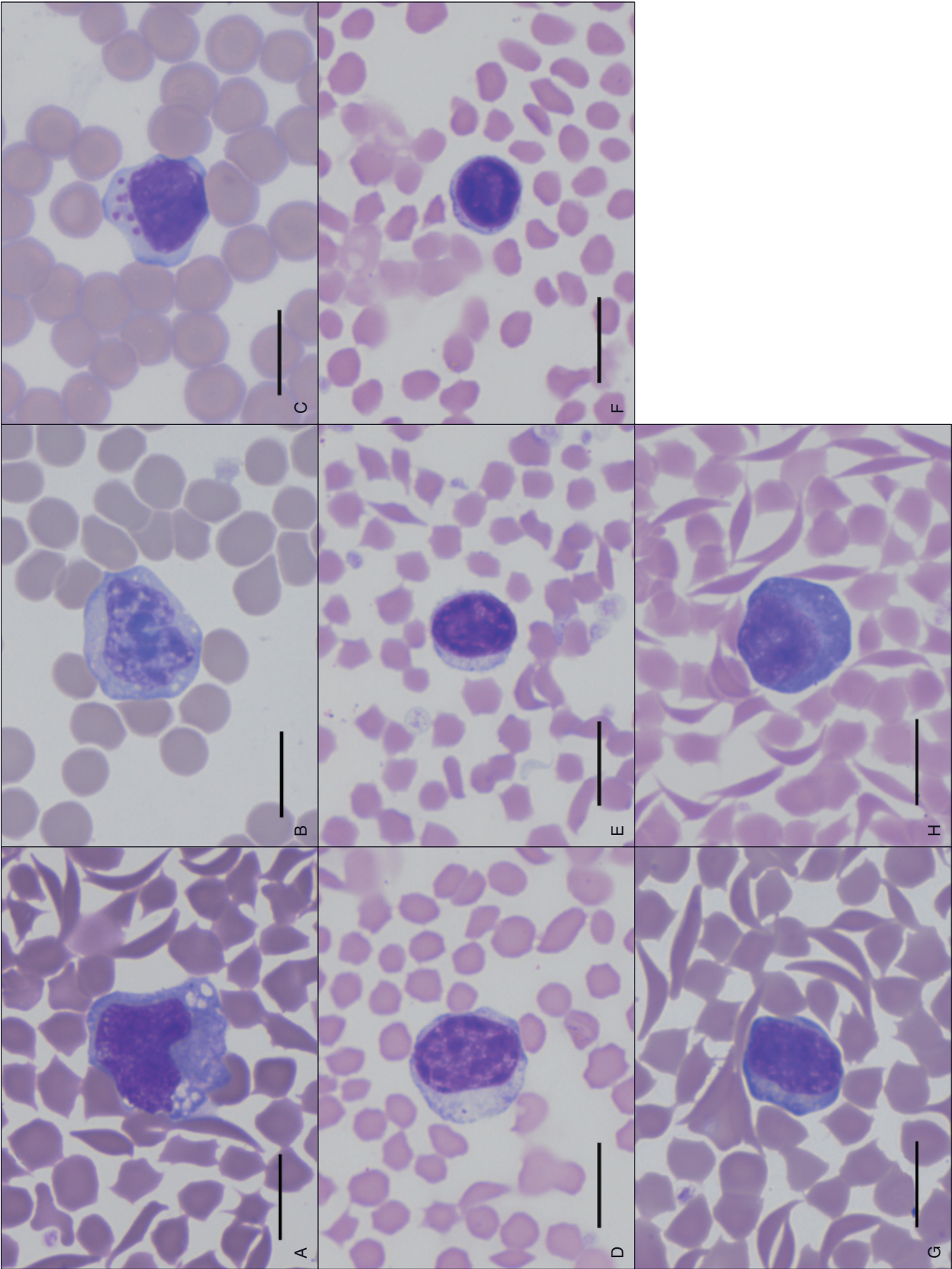


FIGURE 116.4 Normal circulating cervid monocytes and lymphocytes. Modified Wright stain; bar = 10 μ m. (A) Deer, monocyte. (B) Wapiti (elk), monocyte. (C) Wapiti (elk), large granular lymphocyte. (D) Deer, large lymphocyte. (E) Deer, medium-sized lymphocyte. (F) Deer, small lymphocyte. (G) Deer, reactive lymphocyte with basophilic cytoplasm. (H) Wapiti (elk), reactive lymphocyte.

Platelets

The platelets (PLT) of cervids are relatively small (Fig. 116.1A), and the reference interval is variable between different studies.^{13,22,29} PLT values in physically restrained and sedated wild red deer are $344 \pm 138 \times 10^3$ and $311 \pm 117 \times 10^3$ PLT/ μ L, respectively.²² Wild, physically restrained tule wapiti have PLT counts of $310 \pm 76.8 \times 10^3$ PLT/ μ L, with a range of $43\text{--}581 \times 10^3$ PLT/ μ L.²⁹

REFERENCE INTERVALS

Complete blood count data from various species of deer and wapiti are depicted in Tables 116.1 and 116.2, which summarize data acquired through impedance cytometry. Data from older methodologies that utilize the hemocytometer can be found in the previous editions of this book.

Within the Cervidae, interspecies variations of “normal” hematology exist. White-tailed deer have RBC, Hgb, and packed cell volume (PCV) values that are considerably higher than those found in mule deer, black-tailed deer, or chital deer.³¹ Inversely, the leukocyte count of white-tailed deer tends to be slightly lower than that reported for other species of deer.³¹

Influence of Age

Neonate and juvenile deer erythrocytes have less of a tendency to sickle. This is due to the presence of fetal hemoglobin, which precludes sickling.¹⁷ At birth, 95% of the total hemoglobin is fetal hemoglobin. By 8–12 weeks of age, the fetal hemoglobin is completely replaced by adult hemoglobin.¹⁷ At this age, sickling may be readily observed if the appropriate hemoglobin forms are present.

There is a distinct and direct relationship between the RBC count, Hgb, and PCV and age, regardless of sex or species.^{4,21} Newborn animals have lower RBC values and higher reticulocyte counts than juveniles and adults, and juveniles tend to have lower RBC values than adults.^{4,5} Increases in RBC count and hematocrit (Hct) by 40–60% from birth to 10 months of age were noted in chital deer.⁴ Similar findings have been reported for captive reindeer, fallow deer, and white-tailed deer.^{3,4,27,36}

There is an unclear relationship between age and white blood cell (WBC) parameters in deer. While some studies have reported wide WBC reference intervals for neonates, or failed to demonstrate a relationship between age and WBC counts, other studies have shown a correlation.^{16,25,27,32} Chapple determined that the total WBC count in neonatal chital deer was significantly greater than that of juveniles or adults, largely due to increased numbers of neutrophils and a neutrophil:lymphocyte ratio of 2:1.⁴ In adult chital deer, lymphocytes predominate.⁴

Influence of Gender

Gender-related changes in hematologic patterns are not apparent in neonates or juveniles but may be evident in adults. Chital stags have higher RBC values than females after 140 days of age. Red cell parameters were 15–22% higher in chital stags than in chital hinds, but the erythrocyte indices were lower in the stags than in the hinds. The total WBC count was significantly higher in stags.⁴ Similar patterns have been noted in male and female rusa deer and farmed fallow deer.^{2,36} However, this observation does not appear to be absolute, since several studies did not observe these patterns.^{1,22}

Influence of Reproductive Status

Complete blood count changes due to reproductive status are seen in both male and female cervids. During the annual rutting period, chital deer stags have altered differential leukocyte counts, and red deer stags have their lowest mean RBC count.^{4,5,36} Pregnancy results in significant increases in Hgb, RBC count, Hct, and mean corpuscular volume (MCV) with a decrease in mean corpuscular hemoglobin concentration (MCHC).^{29,36} The WBC count also increases due to increased numbers of lymphocytes. Lactating does may have lower red cell numbers.³⁶

Influence of Stress and Handling

Deer are easily excitable as a species, which is often reflected in the hematologic parameters. Red and white cell values are significantly higher in excited deer than in resting or chemically restrained deer. This is likely due to a combination of drug-specific effects in the anesthetized deer and a greater stress response, and subsequent splenic contraction, in the physically restrained deer.^{21,22,30} Erythrocyte and leukocyte parameters may differ between the sexes in response to handling and restraint. This effect is most appreciable in chital does, resulting in significantly greater increases in PCV and red and white cell numbers than seen in chital bucks.⁴ However, little or no difference in WBC count may be seen between excited and resting males, or between farmed male and wild female deer.²¹

Periodic handling of cervids may reduce handling stress, and subsequently impact hematologic parameters. One study observed that regularly handled hinds had reductions in their Hgb, RBC count, and Hct from 10% to 20% over time.⁴ Stags did not show these distinct and sequential erythron changes with handling.^{4,21} However, they did have an altered differential leukocyte count during initial sampling periods. The annual male reproductive cycle may have confounded hematologic alterations associated with adaptation to handling stress.⁴

Response to Disease

The hematologic response in deer has been evaluated in response to experimental infection with numerous

TABLE 116.1 Hematologic Values of Deer

Reference	Animal	No., Gender ^a (Age)	RBC ($\times 10^6/\mu\text{L}$)	Hgb (g/dL)	Hct or PCV (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	WBC ($\times 10^3/\mu\text{L}$)	Differential Leukocyte Count ($\times 10^3/\mu\text{L}$)				
										Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils
5	Chital ^{b,c,d} (Australia)	3 M, 5 F (1–2 d)	10.22 ± 1.60	10.90 ± 1.13	34 ± 4	33.15 ± 4.60	10.94 ± 1.40	32.7 ± 0.9	8.40 ± 2.60	5.810 ± 1.740	2.320 ± 1.290	0.174 ± 0.088	0.085 ± 0.103	0
5	Chital ^{b,c,d,e} (Australia)	1 F (<1 yr)	14.25	16.48	46	30.85	11.47	35.8	4.30	1.780	2.252	0.117	0.134	0
5	Chital ^{b,c,d,e} (Australia)	1 M (<1 yr)	16.48	17.12	46	27.70	10.38	37.4	4.52	1.373	3.119	0.098	0.104	0.006
5	Chital ^{b,c,d,e} (Australia)	10 F (>2 yr)	11.64 ± 0.72	14.46 ± 6.6	39 ± 2	33.68 ± 2.58	12.47 ± 0.91	36.8 ± 0.7	3.92 ± 0.44	1.673 ± 0.335	1.946 ± 0.377	0.136 ± 0.060	0.181 ± 0.086	0.003 ± 0.008
5	Chital ^{b,c,d,e} (Australia)	10 F (pregnant, >2 yr)	13.28 ± 0.80	16.43 ± 0.53	47 ± 1	35.45 ± 2.61	12.43 ± 0.88	35.1 ± 0.7	4.87 ± 1.08	1.766 ± 0.265	2.741 ± 0.307	0.168 ± 0.073	0.162 ± 0.109	0.001 ± 0.008
5	Chital ^{b,c,d,e} (Australia)	7 M (>1 yr)	14.27 ± 0.34	16.54 ± 0.65	46 ± 1	31.97 ± 1.46	11.62 ± 0.64	36.3 ± 1.5	4.41 ± 0.44	1.738 ± 0.548	2.385 ± 0.594	0.085 ± 0.020	0.127 ± 0.042	0.001 ± 0.006
34	Fallow ^{b,d,f} (Slovenia)	43 MF (>6 mo)	9.8 ± 1.7	14.5 ± 2.1	44.3 ± 7.2	45.3 ± 3.8	14.9 ± 1.5	32.9 ± 1.5	2.65 ± 0.9	0.54 ± 0.28	1.92 ± 0.72	0.02 ± 0.03	0.15 ± 0.12	0.02 ± 0.03
35	Red ^{b,c} (New Zealand)	90–91 MF	—	16.0 (CL = 4.2) ^j	44.6 (CL = 11.8) ^j	—	—	35.8 (CL = 3.9) ^j	—	—	—	—	—	—
22	Red ^{c,d,g} (Spain)	20 F (1.5–9 yr)	13.6 ± 2.38	18.7 ± 1.6	54.4 ± 8.5	40.7 ± 6.9	14.1 ± 2.1	34.9 ± 4.2	5.51 ± 1.92	3.52 ± 1.88	1.75 ± 0.70	0.23 ± 0.17	0.05 ± 0.09	0
22	Red ^{d,g,h} (Spain)	9 M, 11 F (1.5–9 yr)	10.9 ± 2.0	15.9 ± 2.4	44 ± 7.7	40.5 ± 4.1	15.8 ± 5.0	35.1 ± 5.3	2.97 ± 1.05	1.59 ± 0.97	1.04 ± 0.41	0.11 ± 0.08	0.18 ± 0.11	0.01 ± 0.03
32	Rusa ^{b,c,e,i} (Australia)	8 M (<90 d)	7.7 ± 0.28 (4.2–10.2)	11.7 ± 0.57 (6.0–17.4)	35.0 ± 0.92 (26.0–46.0)	48.7 ± 2.14 (35.2–73.8)	17.1 ± 0.82 (10.3–26.4)	32.8 ± 0.94 (23.3–43.6)	6.5 ± 0.55 (2.9–21.0)	—	—	—	—	—
32	Rusa ^{b,c,e,i} (Australia)	8 M (90–180 d)	8.7 ± 0.31 (5.8–11.7)	12.0 ± 0.40 (7.9–17.4)	37.3 ± 0.57 (33.0–46.0)	44.6 ± 1.63 (30.7–61.5)	14.3 ± 0.68 (10.2–25.6)	32.3 ± 0.97 (24.0–42.7)	6.3 ± 0.25 (4.4–11.2)	—	—	—	—	—
32	Rusa ^{b,c,e,i} (Australia)	8 M (>180 d)	6.2 ± 0.18 (4.2–8.7)	13.3 ± 0.59 (6.3–20.9)	35.0 ± 1.21 (16.0–47.0)	58.1 ± 1.40 (42.2–78.6)	21.9 ± 0.96 (13.4–37.5)	37.6 ± 1.26 (25.1–58.6)	7.0 ± 0.23 (4.4–12.4)	—	—	—	—	—
25	White-tailed ^{c,g,i} (Minnesota)	22 MF (0–1 d)	7.8 ± 0.3 (5.6–10.7)	8.5 ± 0.3 (6.1–11.4)	29.8 ± 1.2 (21.0–42.0)	38.2 ± 0.5 (32.0–43.0)	10.9 ± 0.2 (8.0–13.0)	28.5 ± 0.4 (26.0–33.0)	3.1 ± 0.3 (1.0–6.2)	—	—	—	—	—
25	White-tailed ^{c,g,i} (Minnesota)	18–20 MF (0–7 d)	7.8 ± 0.2 (6.0–9.2)	7.9 ± 0.2 (5.9–8.9)	27.6 ± 1.0 (19.0–34.0)	35.2 ± 0.8 (29.0–41.0)	9.8 ± 0.2 (8.0–11.0)	27.7 ± 0.5 (24.0–33.0)	2.6 ± 0.2 (1.1–4.3)	—	—	—	—	—
28	White-tailed ^{d,f,g} (Minnesota)	33 F (>1 yr)	11.2 ± 1.6	14.7 ± 2.6	44.0 ± 7.4	39 ± 3.7	—	33.6 ± 1.3	—	—	—	—	—	—
28	White-tailed ^{d,f,g} (Minnesota)	18 M (>1 yr)	10.8 ± 1.2	13.5 ± 1.9	40.0 ± 5.7	37 ± 3.5	—	33.7 ± 1.2	—	—	—	—	—	—

^aF, female; M, male; MF, males and females.

^bCaptive animals.

^cPhysically restrained animals.

^dResults reported as mean ± standard deviation (range when available).

^eSerial sampling of the same animals.

^fShot animals.

^gWild animals.

^hChemically restrained animals.

ⁱResults reported as mean ± standard error (range when available).

^jCL, 95% confidence limit.

TABLE 116.2 Hematologic Values of Wapiti (Elk)

Reference	Animal	No., Gender ^a (Age)	RBC ($\times 10^6/\mu\text{L}$)	Hgb (g/dL)	Hct or PCV (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	WBC ($\times 10^3/\mu\text{L}$)	Differential Leukocyte Count ($\times 10^3/\mu\text{L}$)				
										Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils
14	Rocky Mountain ^{b,c,d} (Colorado)	31 MF	—	18.3 \pm 0.808	46.3 \pm 2.12	—	—	—	—	—	—	—	—	—
24	Rocky Mountain ^{c,d,e} (Oregon)	33 MF (>1 yr)	10.97 \pm 1.00 (9.36–13.20)	19.03 \pm 1.58 (16.2–22.0)	67.62 \pm 5.74 (58.0–76.5)	62.02 \pm 2.40 (57.5–65.0)	—	—	8.628 \pm 2.493 (6.500–14.600)	—	—	—	—	—
29	Tule ^{b,c,d} (California)	94 F, 5 M (>1 yr)	9.83 \pm 0.99 (6.57–13.98)	16.29 \pm 1.29 (9.70–20.10)	48.10 \pm 3.67 (28.0–60.60)	49.55 \pm 3.55 (39.50–63.30)	16.61 \pm 1.32 (12.40–21.40)	33.42 \pm 1.34 (13.00–36.10)	8.07 \pm 1.58 (4.40–15.9)	2.96 \pm 1.14 (0.552–6.431)	3.55 \pm 1.06 (0.435–7.104)	0.384 \pm 0.201 (0.044–1.030)	0.898 \pm 0.458 (0.171–2.584)	0.100 \pm 0.088 (0–0.450)

^aF, female; M, male; MF, males and females.

^bPhysically restrained animals.

^cResults reported as mean \pm standard deviation (range when available).

^dWild animals.

^eChemically restrained animals.

infectious agents such as epizootic hemorrhagic disease virus, bluetongue virus, and *Yersinia pseudotuberculosis*.^{6,15,26} Lymphopenia was a common response. One wapiti infected with *Babesia odocoilei* presented with a normocytic normochromic non-regenerative anemia and an inflammatory leukogram, characterized by a leukopenia and neutropenia.⁸

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Hematology of Water Buffalo (*Bubalia bubalis*)

TAMARA B. WILLS

Blood

- Erythrocytes
- Leukocytes
 - Neutrophils
 - Lymphocytes
 - Monocytes
 - Eosinophils
 - Basophils

Platelets

Plasma Fibrinogen

Reference Intervals

Age Related, Physiologic, Environmental, and
Miscellaneous Influences Affecting the
Hemogram

Acronyms and Abbreviations

ESR, erythrocyte sedimentation rate; MCV, mean corpuscular volume; PCV, packed cell volume; RBC, red blood cell; WBC, white blood cell.

BLOOD

Features of a normal water buffalo hemogram are comparable to cattle with minor variations. The morphology of the red blood cells (RBCs), white blood cells (WBCs), and platelets is similar to cattle. Differences include a faster erythrocyte sedimentation rate (ESR), lower icteric index, and greater rouleaux formation in water buffalo compared to cattle.^{3,6} Tables 117.1 and 117.2 summarize reported hematology values in the literature of water buffalo, including comparisons with respect to age, sex, pregnancy, and lactation.

Erythrocytes

Buffalo RBCs are similar to those of cattle with a mean cell size of $5.5 \pm 0.7 \mu\text{m}$. Red blood cells in normal water buffalo exhibit mild anisocytosis and no significant polychromasia. Only rare Howell-Jolly bodies are found and prominent central pallor is not observed. Reticulocytes are not typically present in normal water buffalo.^{2,3} Unlike cattle, slight to marked rouleaux formation is a frequent finding.^{3,6}

Leukocytes

Neutrophils

Neutrophils have a mean size of $13.2 \pm 0.7 \mu\text{m}$ and are multilobed or occasionally monolobed. Multilobed neu-

trophils have an irregular nuclear outline with 2–4 lobes and a dense chromatin pattern.³ Monolobed nuclei have 1–2 constrictions, with decreased amounts of condensed chromatin compared to multilobed neutrophils. The cytoplasm is pale with fine indistinct, sometimes brown granules.^{2,3} Band neutrophils are rarely found in normal water buffalo, but have a smooth nuclear outline with no nuclear constrictions.³

Lymphocytes

Similar to cattle, small, medium, and large lymphocytes are present in varying numbers, although one size frequently predominates in a single animal. The average size is $11.1 \pm 1.8 \mu\text{m}$.² The nuclei range from round to oval and sometimes contain a slight indentation. The nuclear pattern is frequently coarsely granular, although it appears slightly more homogeneous in larger lymphocytes. Occasional lymphocytes also contain nucleolar rings. The cytoplasm ranges from pale to basophilic and sometimes contains eosinophilic granules or clear vacuoles.³ Larger lymphocytes can sometimes be difficult to differentiate from monocytes.² Similar to sheep, goats, and cattle, lymphocyte numbers frequently exceed neutrophils at approximately 1.5:1.³

Monocytes

Monocytes are typically larger than lymphocytes, approximately $15.4 \pm 1.5 \mu\text{m}$ in size, with an amoeboid

TABLE 117.1 Hemogram Values of Water Buffalo with respect to Age, Pregnancy, Lactation, and Gender

	PCV (%)	Hgb (g/dL)	RBCs ($\times 10^6/\mu\text{L}$)	MCV (fL)	MCHC (g/dL)	MCH (pg)
Age Group						
Day old ^a	42.65 \pm 3.74	14.16 \pm 0.59	7.01 \pm 0.91	58.25 \pm 6.54	41.26 \pm 3.99	26.51 \pm 5.69
1 month ^a	26.66 \pm 5.25	11.06 \pm 0.65	4.11 \pm 1.64	84 \pm 5.58	44.63 \pm 3.97	29.49 \pm 11.15
0–3 months ^b	45.1 \pm 6.5	14.98 \pm 1.5	8.35 \pm 0.31	54.01 \pm 3.91	33.15 \pm 3.85	17.94 \pm 1.80
3–6 months ^b	36.80 \pm 3.80	11.98 \pm 1.98	6.50 \pm 0.70	56.6 \pm 4.36	32.55 \pm 4.5	18.43 \pm 2.15
6 months ^a	42.5 \pm 1.98	13.48 \pm 0.28	7.74 \pm 0.73	52.71 \pm 1.61	34.44 \pm 2.32	18.55 \pm 1.86
12 months ^a	33.78 \pm 2.83	10.35 \pm 0.78	5.56 \pm 0.62	61.8 \pm 7.11	33.98 \pm 2.93	19.23 \pm 2.12
20–24 months ^a	41.41 \pm 0.44	14.23 \pm 0.51	7.46 \pm 0.62	67.11 \pm 0.10	32.71 \pm 0.02	24.49 \pm 0.04
25–30 months ^a	39.08 \pm 0.74	13.29 \pm 0.42	7.1 \pm 0.72	66.75 \pm 5.25	33.46 \pm 1.03	24.02 \pm 2.23
24–48 months ^b	39.80 \pm 3.79	12.10 \pm 1.36	7.8 \pm 0.38	51.02 \pm 3.82	30.4 \pm 3.06	15.51 \pm 2.80
Gender, Pregnancy, and Lactation						
Immature females, 6 months to 2 years ^c	33 \pm 5	11.5 \pm 1.62	7.2 \pm 1.4	46.1 \pm 4.5	35.1 \pm 1.8	16 \pm 1.4
Adult female ^b	40.2 \pm 6.80	12.78 \pm 0.85	7.20 \pm 0.90	55.83 \pm 3.09	31.79 \pm 1.76	17.75 \pm 1.08
Adult female ^d	30.58 \pm 0.25	11.81 \pm 0.15	6.77 \pm 0.08	45.63 \pm 0.50	38.95 \pm 0.60	17.70 \pm 0.30
Adult female pregnant ^d	32.22 \pm 0.28	12.22 \pm 0.24	7.06 \pm 0.07	45.92 \pm 0.30	37.67 \pm 0.40	17.21 \pm 0.20
Adult female lactating ^e	26–34	9–13.5	5.07–8.27	40.6–55.2	20.9–38.5	13.5–20.5
Immature males, 6 months to 2 years ^c	39 \pm 5	14.11 \pm 1.97	7.9 \pm 1.2	50.2 \pm 6.1	36.34 \pm 2.1	18 \pm 1.7
Adult bull ^b	41.2 \pm 8.26	13.5 \pm 0.95	6.95 \pm 0.73	59.28 \pm 4.56	32.76 \pm 3.01	19.42 \pm 1.32

^aRef. 7.^bRef. 10.^cRef. 2.^dRef. 9.^eRef. 3.

nucleus and a lacy to granular chromatin pattern.² The cytoplasm is basophilic, frequently containing vacuoles, although sometimes it appears granular. Azurophilic granules are occasionally noted in the cytoplasm.³

Eosinophils

Eosinophils are approximately $13.9 \pm 0.9 \mu\text{m}$ in size and are less lobulated, with a smoother nuclear outline than neutrophils.² The cytoplasm contains uniform, round, bright pink granules which fill the pale cytoplasm.³

Basophils

Basophils are approximately $13.4 \pm 1.0 \mu\text{m}$ in size and contain numerous purple round to rod shaped granules that frequently obscure the nucleus.²

Platelets

Platelets are abundant and present individually as well as in clumps. Platelets range from round to elongate in

shape and frequently contain distinct, variably sized purple granules. Platelet size ranges from 2.4 to $3.0 \mu\text{m}$ but can be larger.^{2,3}

Plasma Fibrinogen

Fibrinogen levels are lower in water buffalo calves compared to 6 month old and adult water buffaloes. One study reported that 2 week old water buffalo calves had a mean plasma fibrinogen level of 530 mg/dL compared to 6 month old buffaloes with a mean plasma fibrinogen concentration of 820 mg/dL.⁸ Other reported fibrinogen levels are $513 \pm 62 \text{ mg/dL}$, $615 \pm 90 \text{ mg/dL}$, and $544 \pm 74 \text{ mg/dL}$ for buffalo calves, lactating buffaloes, and non-lactating buffaloes, respectively.⁵

REFERENCE INTERVALS

The impact of age, environment, corticosteroid administration, response to endotoxin, and some miscellaneous environmental conditions on the hemogram have been reported.

TABLE 117.2 Leukogram Values for Water Buffalo with respect to Age, Pregnancy, Lactation, and Gender

	Leukocytes ($\times 10^3/\mu\text{L}$)	Bands (%)	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)	Basophils (%)
Age Group							
Day old ^a	8.22 \pm 1.49	NR	47.16 \pm 3.3	50.16 \pm 3.69	2 \pm 0.05	2 \pm 0.57	NR
1 month ^a	16.06 \pm 1.06	NR	33 \pm 1.63	64 \pm 1.69	1 \pm 0.47	2 \pm 0.47	NR
0–3 months ^b	5.90 \pm 0.10	NR	35.10 \pm 3.50	50.90 \pm 6.50	6.50 \pm 0.95	7.50 \pm 1.10	0 \pm 0
3–6 months ^b	6.85 \pm 0.15	NR	32.25 \pm 4.5	54.15 \pm 8.90	6.25 \pm 0.60	6.35 \pm 1.08	1 \pm 0.01
6 months ^a	12.6 \pm 1.35	NR	26 \pm 6.67	68.66 \pm 6.4	2 \pm 0	2.3 \pm 0.27	NR
12 months ^a	17.08 \pm 3.54	NR	31.44 \pm 3.48	66.22 \pm 3.63	0.11 \pm 0.1	1.44 \pm 0.27	NR
20–24 months ^a	12.69 \pm 1.65	NR	29.55 \pm 1.99	67.07 \pm 1.99	0.98 \pm 0.2	2.3 \pm 0.38	NR
25–30 months ^a	13.15 \pm 1.52	NR	31.05 \pm 3.06	66.17 \pm 3.66	1.09 \pm 0.12	2.42 \pm 0.38	NR
24–48 months ^b	6.10 \pm 0.23	NR	30.75 \pm 4.86	57.25 \pm 5.20	6.50 \pm 0.30	5.50 \pm 0.36	0 \pm 0
Sex, Pregnancy, Lactation							
Immature females, 6 months to 2 years ^c	10.7 \pm 2.2	0.1 \pm 0.4	36.1 \pm 9.4	50.4 \pm 9.2	8.7 \pm 4.2	3.4 \pm 3.9	1.5 \pm 1.5
Adult female ^b	6.89 \pm 0.30	NR	28.70 \pm 3.83	60.3 \pm 3.55	7.5 \pm 0.31	3.5 \pm 0.15	0 \pm 0
Adult female ^d	NR	NR	NR	NR	NR	NR	NR
Adult female, pregnant ^d	NR	NR	NR	NR	NR	NR	NR
Adult female, lactating ^e	6.25–13.05	0–1	13–54	26–75	1–11.5	2–14.0	0–3.5
Immature males, 6 months to 2 years ^c	10.2 \pm 2.5	0.1 \pm 0.3	35 \pm 8.8	52 \pm 9.1	6.2 \pm 2.6	6.7 \pm 4.2	0.1 \pm 0.2
Adult bull ^b	6.53 \pm 0.18	NR	25.35 \pm 2.28	63.65 \pm 8.28	8 \pm 0.4	3 \pm 0.12	0 \pm 0

NR, not reported.

^aRef. 7.^bRef. 10.^cRef. 2.^dRef. 9.^eRef. 3.

Age Related, Physiologic, Environmental, and Miscellaneous Influences Affecting the Hemogram

Hematologic differences with respect to age include differences in the packed cell volume (PCV), mean corpuscular volume (MCV), ESR, and the neutrophil to lymphocyte ratio. For example, the PCV of water buffalo decreases between 1 and 3 months of age and increases gradually to 6 months, stabilizing at approximately 24 months of age (Table 117.1). Total leukocyte counts are also slightly lower in newborns compared to adults (Table 117.2).^{7,10} The MCV increased in one study at 1 month of age with a concurrent decrease in PCV and then decreased to adult values.⁷ A separate study reported a similar change in MCV in young calves 0–3 months of age compared to adults.¹⁰ The variations in MCV reported in the literature may be affected by the environment, parasite control, and differences in nutrition and husbandry. The neutrophil to lymphocyte ratio

decreases gradually from birth to adulthood due to a decrease in neutrophil numbers and a gradual increase in lymphocytes.^{7,10} The ESR increases gradually and is highest in adults and is associated with a concurrent increase in whole blood viscosity.⁸

Leukocyte responses due to corticosteroid injection have been evaluated in buffalo calves. A single injection of corticosteroid, either prednisolone or dexamethasone, resulted in a leukocytosis with a neutrophilia and no left shift, lasting from 2 to 24 hours. Prednisolone administration also caused an initial monocytosis 2–8 hours post-administration and an eosinopenia and basopenia by 6 hours post-administration. Interestingly, a lymphopenia was not evident after prednisolone administration. Inconsistent changes were noted in lymphocyte, monocyte, eosinophil, and basophil numbers after dexamethasone administration. The lack of a lymphopenia with steroid administration is dissimilar to many other species.⁴

Very little research has been performed to evaluate the impact of the environment on the water buffalo hemogram. Spray cooled and wallowing buffalo had lower RBC numbers, hemoglobin concentration, and PCV compared to those in normal environmental temperature.¹

Other miscellaneous influences on the water buffalo hemogram include gender, diurnal variations, and response to endotoxin. Differences between males and females have been reported, the most significant being that males frequently have increased hemoglobin content and sometimes PCV compared to females (Table 117.1).¹⁰ Diurnal variations in leukocyte numbers have also been suggested in water buffalo calves.⁴

Hematologic changes are observed in response to endotoxin injection and are dose dependent. Low dose endotoxin injection (10 µg) resulted in mild leukopenia and mild lymphopenia and a moderate neutrophilia 6 hours post-administration. A trend toward a more significant leukopenia, neutropenia, and lymphopenia was noted with higher doses of endotoxin. A rebound neutrophilia was not observed after endotoxin administration, although numbers of neutrophils returned to normal by 6 hours for endotoxin doses of 50 µg or less and within 24 hours for doses of 100 µg endotoxin.⁴

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Hematology of the American Bison (*Bison bison*)

KURT L. ZIMMERMAN, DAVID M. MOORE, and STEPHEN A. SMITH

Blood Collection
Morphology and Numbers of Peripheral Blood Cells
Erythrocytes

Leukocytes
Reference Interval Influences

Acronyms and Abbreviations

CBC, complete blood count; Hgb, hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PCV, packed cell volume; RBC, red blood cell; WBC, white blood cell.

BLOOD COLLECTION

Venipuncture sites for blood collection in the American Bison (*Bison bison*) include the jugular vein and coccygeal (tail) vein, using the same techniques that would be used in domestic cattle. Blood collection can be accomplished in an unanesthetized animal when restrained within a squeeze chute.

MORPHOLOGY AND NUMBERS OF PERIPHERAL BLOOD CELLS

Reported hematologic values for American Bison are provided in Tables 118.1 and 118.2.^{1,2,4,7,13,14,17,19,21,22} The morphology of erythrocytes, leukocytes, and platelets (Figs. 118.1, 118.2, 118.3, 118.4) in bison is comparable to that observed in domestic cattle (*Bos taurus*).^{1,2,11,13–15,17,19,21,22} These hematologic morphological similarities with domestic cattle extend to the presence of similar red-cell antigens (A, B, C and F of various systems).^{3,16} However, unique red cell antigens and hemoglobin types are also recognized.^{3,10,20}

Erythrocytes

The average packed cell volume (PCV) in bison is approximately 47% (range, 24–61%). Compared with

reported values for domestic cattle, sheep, and goats, the following parameters are higher in bison: red blood cell (RBC) counts, PCV, and the hemoglobin (Hgb) concentration. The average Hgb in bison is about 16.8 g/dL (range, 8.2–23.0 g/dL).^{1,2,11,13,14,19,21,22}

Leukocytes

The following leukocyte parameters are comparable with reported values in domestic cattle, sheep, and goats: white blood cell count (WBC), neutrophil count, lymphocyte count, eosinophil count, and monocyte count.¹⁸ Lymphocytes outnumber neutrophils in both bison and domestic cattle. This is in contrast to earlier reports that indicated that the neutrophil:lymphocyte ratio in bison was opposite to that observed in cattle.^{14,19} The average total leukocyte count for all ages and sexes of bison is $8.0 \times 10^6/\mu\text{L}$ (range, $1.0 \times 10^6/\mu\text{L}$ to $18.4 \times 10^6/\mu\text{L}$).^{1,2,11,13,14,19,21,22}

Reference Interval Influences

Bison calves have significantly higher RBC counts than do adult bison, which is also the case with calves of domestic cattle.¹⁸ Average oxygen-carrying capacity was 17.0 mL/100 mL from calf blood and 22.2 mL/100 mL blood from adult blood; Hgb concentration averaged

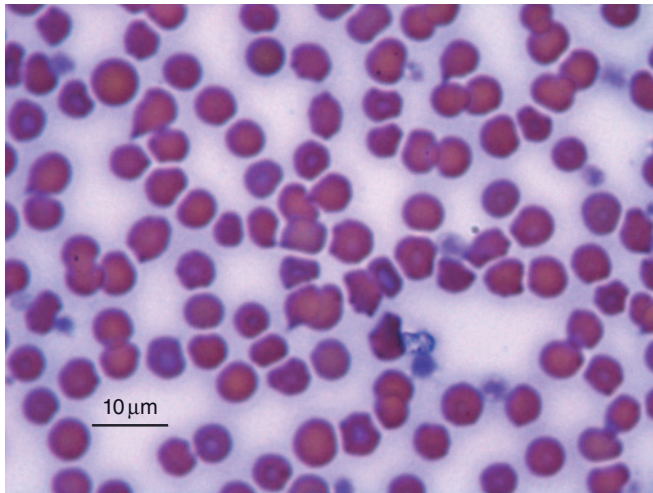


FIGURE 118.1 Erythrocytes and platelets of the American Bison (*Bison bison*). 100× objective; Wright's stain.

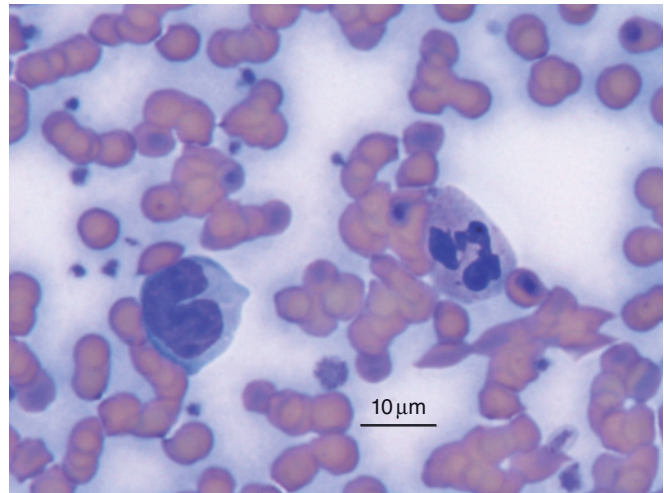


FIGURE 118.3 Monocyte and neutrophil of the American Bison (*Bison bison*). 100× objective; Wright's stain.

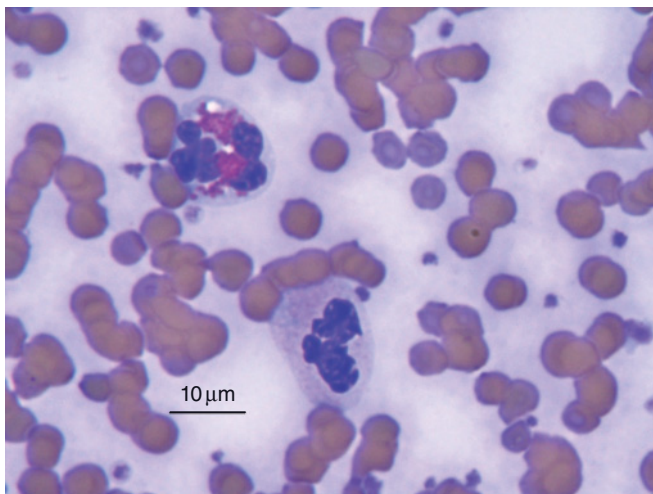


FIGURE 118.2 Neutrophil and eosinophil of the American Bison (*Bison bison*). 100× objective; Wright's stain.

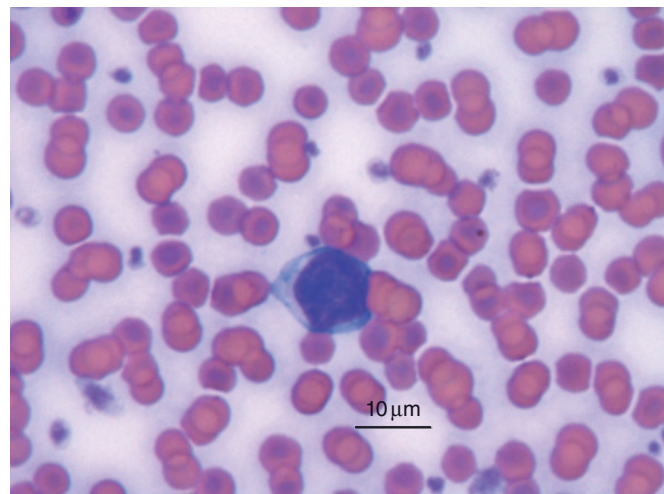


FIGURE 118.4 Lymphocyte of the American Bison (*Bison bison*). 100× objective; Wright's stain.

13.6 g/dL in calves and 17.1 g/dL in adults.⁶ Other age related variations in bison complete blood count (CBC) results include: RBC, monocyte, hematocrit, and neutrophil numbers which are negatively correlated, while lymphocyte numbers are positively correlated with increasing age.¹

Bison raised and/or maintained under ranch conditions comparable to those used in beef cattle operations have similar hematologic values as free-ranging bison living on range lands or in national parks.¹⁹ However, an increase in the PCV is seen in free-ranging

wild bison impounded for 24 hours before blood collection.⁷ Seasonal variation in the PCV, mean corpuscular hemoglobin concentration (MCHC), mean cell volume (MCV), and Hgb values are observed and may be a factor in establishing reference intervals.⁴

Variation of hematologic values from those summarized above have been specifically reported in bison in association with infection with *Trypanosoma theileria* and *T. evansi*, *Theileria annulata*, *Mycobacterium bovis*, and Degnala disease.^{5,8,9,12,15}

TABLE 118.1 Referenced Erythrocyte Parameters of the American Bison (*Bison bison*)

Reference	Environment	Age	Gender	RBC ($\times 10^6/\mu\text{L}$)		PCV (%)		Hgb (g/dL)		MCV (fL)		MCH (pg)		MCHC (%)	
				Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range
13		<2yr	NS	—	—	49.8 \pm 1.3	—	16.6 \pm 1.3	12.5–19.0	—	—	—	—	—	—
		Adult	NS	—	—	50.0 \pm 4.5	42.6–61.1	17.2 \pm 1.5	14.2–21.0	—	—	—	—	—	—
14		All	M/F	10.08 \pm 1.43	6.53–13.9	47.11 \pm 4.06	37–57	16.99 \pm 1.43	12.24–19.19	59.74 \pm 8.76	41.01–87.29	19.79 \pm 4.4	11.42–29.38	35.92 \pm 6.21	21.47–51.86
		All	F	10.33 \pm 1.45	—	47.2 \pm 4.46	—	16.93 \pm 1.47	—	45.69 \pm 9.41	—	16.39 \pm 4.53	—	35.87 \pm 5.34	—
		1–2yr	F	10.14 \pm 1.41	—	46.95 \pm 4.99	—	17.06 \pm 1.6	—	46.3 \pm 7.56	—	16.82 \pm 2.96	—	36.34 \pm 3.29	—
		3–4yr	F	10.01 \pm 1.67	—	48.52 \pm 3.29	—	16.97 \pm 1.04	—	48.47 \pm 8.63	—	16.95 \pm 3.63	—	34.97 \pm 4.56	—
		> 5yr	F	10.97 \pm 1.12	—	46.14 \pm 4.03	—	16.52 \pm 1.49	—	42.06 \pm 8.97	—	15.06 \pm 3.97	—	35.8 \pm 4.99	—
		All	M	9.97 \pm 1.32	—	46.9 \pm 3.73	—	16.72 \pm 1.34	—	47.0 \pm 9.73	—	16.77 \pm 4.38	—	35.65 \pm 4.71	—
		1–2yr	M	9.64 \pm 1.28	—	46.17 \pm 4.27	—	16.51 \pm 1.58	—	47.89 \pm 8.43	—	17.17 \pm 3.59	—	35.76 \pm 4.87	—
		3–4yr	M	9.93 \pm 1.28	—	47.63 \pm 3.49	—	16.93 \pm 1.24	—	47.97 \pm 7.56	—	18.15 \pm 4.13	—	35.54 \pm 3.96	—
		> 5yr	M	11.24 \pm 0.92	—	42.44 \pm 8.47	—	17.0 \pm 0.85	—	42.44 \pm 8.47	—	15.13 \pm 4.53	—	35.63 \pm 5.11	—
11	Winter	NS	M/F	—	—	43 \pm 4	—	15.8 \pm 1.1	—	—	—	—	—	—	—
	Spring	NS	M/F	—	—	41.7 \pm 2.5	—	15.2 \pm 0.6	—	—	—	—	—	—	—
	Summer	NS	M/F	—	—	26.8 \pm 3.1	—	16.7 \pm 1.4	—	—	—	—	—	—	—
	Fall	NS	M/F	—	—	51.4 \pm 4.2	—	18.6 \pm 1.3	—	—	—	—	—	—	—
7	Ranched	NS	NS	8.5 \pm 0.5	—	46.8 \pm 2.6	—	17.5 \pm 0.9	—	55.1 \pm 1.6	—	20.2 \pm 0.4	—	36.5 \pm 0.8	—
	Park 1	Juvenile	NS	8.7 \pm 0.5	—	44.1 \pm 2.0	—	16.7 \pm 0.8	—	50.8 \pm 2.7	—	18.7 \pm 0.8	—	36.7 \pm 0.6	—
	Park 1	Adult	NS	7.9 \pm 0.6	—	45.9 \pm 4.1	—	16.6 \pm 1.4	—	58.5 \pm 4.7	—	20.7 \pm 2.3	—	35.3 \pm 3.6	—
	Park 2	Juvenile	NS	7.9 \pm 1.2	—	39.7 \pm 6.4	—	14.8 \pm 1.5	—	48.7 \pm 2.6	—	19.0 \pm 1.4	—	37.8 \pm 2.1	—
	Park 2	Adult	NS	7.6 \pm 0.8	—	41.3 \pm 3.9	—	15.8 \pm 1.5	—	53.3 \pm 1.9	—	21.1 \pm 0.7	—	38.0 \pm 1.8	—
2		NS	NS	—	—	47.3 \pm 1.3	—	—	—	—	—	—	—	—	—
15		1–2yr	M/F	—	—	51.3 \pm 3.2	28–59	18.6 \pm 1.0	10.3–23.0	—	—	—	—	—	—
19	Ranched	All	M/F	10.19 \pm 1.87	—	44.65 \pm 3.93	—	17.04 \pm 1.62	—	—	—	—	—	—	—
	Ranched	Calf	M/F	11.67 \pm 1.24	—	46.3 \pm 3.69	—	17.59 \pm 1.58	—	—	—	—	—	—	—
				(<185kg)											
	Ranched	Adult	M/F	8.72 \pm 0.84	—	43.0 \pm 3.2	—	16.49 \pm 1.36	—	—	—	—	—	—	—
				(>185kg)											
21		< 6mo	M/F	11.94 \pm 0.25	—	50.77 \pm 1.11	—	17.94 \pm 0.38	—	42.97 \pm 1.07	—	15.04 \pm 0.44	—	35.35 \pm 0.21	—
		7–23mo	M/F	9.64 \pm 0.21	—	50.48 \pm 0.92	—	17.92 \pm 0.31	—	52.37 \pm 0.89	—	18.6 \pm 0.37	—	35.5 \pm 0.18	—
		> 24mo	M/F	9.12 \pm 0.14	—	51.75 \pm 0.61	—	18.36 \pm 0.21	—	56.86 \pm 0.59	—	20.35 \pm 0.24	—	35.49 \pm 0.11	—
22		< 1yr	M/F	9.27 \pm 0.23	5.86–11.38	46.26 \pm 1.42	28–61	16.34 \pm 0.42	9.9–20.9	—	—	—	—	—	—
		Adult	M	8.62 \pm 0.19	5.23–20.7	45.55 \pm 1.21	25–62	15.91 \pm 0.37	8.3–20.7	—	—	—	—	—	—
		Adult	F	8.29 \pm 0.14	5.47–11.13	42.85 \pm 0.68	24–59	15.41 \pm 0.25	8.2–21.2	—	—	—	—	—	—

NS, not specified.

TABLE 118.2 Referenced Leukocyte Parameters of the American Bison (*Bison bison*)

Reference	Environment	Age	Gender	WBC ($\times 10^3/\mu\text{L}$)		Neutrophils (%)		Lymphocytes (%)		Eosinophils (%)		Basophils (%)		Monocytes (%)	
				Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range
13		<2yr	NS	8.0 \pm 2.4	4.0–14.2	34 \pm 10	7–55	56 \pm 5	31–93	7 \pm 5	1–22	0	0–1	2 \pm 2	1–4
		Adult	NS	6.99 \pm 2.1	4.2–13.1	46 \pm 13	22–74	42 \pm 12	22–74	10 \pm 5	2–23	0	0–1	1 \pm 1	0–3
14		All	Both	8.03 \pm 1.41	4.67–10.74	63.77 \pm 7.95	40–83	24.86 \pm 6.4	12–44	3.98 \pm 3.31	0–20	0.77 \pm 1.01	0–10	6.34 \pm 4.17	0–36
		All	F	7.83 \pm 1.6	—	64.03 \pm 8.21	—	24.47 \pm 6.08	—	4.21 \pm 3.81	—	0.77 \pm 1.09	—	6.67 \pm 4.39	—
		1–2yr	F	7.75 \pm 1.69	—	62.67 \pm 9.83	—	25.16 \pm 6.97	—	4.98 \pm 4.79	—	0.86 \pm 1.12	—	6.28 \pm 5.51	—
		3–4yr	F	8.03 \pm 1.39	—	64.14 \pm 6.01	—	24.71 \pm 5.99	—	3.52 \pm 1.94	—	0.31 \pm 0.8	—	7.29 \pm 2.59	—
		>5yr	F	7.8 \pm 1.69	—	66.9 \pm 5.8	—	22.95 \pm 3.96	—	3.38 \pm 2.67	—	1.0 \pm 1.22	—	5.71 \pm 2.65	—
		All	M	8.11 \pm 1.14	—	63.18 \pm 8.1	—	25.35 \pm 7.18	—	3.41 \pm 2.42	—	0.77 \pm 0.97	—	6.64 \pm 4.05	—
		1–2yr	M	8.09 \pm 1.28	—	64.23 \pm 6.64	—	24.14 \pm 6.8	—	3.73 \pm 2.33	—	0.93 \pm 0.91	—	5.33 \pm 3.04	—
		3–4yr	M	8.13 \pm 1.22	—	59.96 \pm 8.96	—	27.81 \pm 7.71	—	2.93 \pm 2.74	—	0.41 \pm 0.75	—	8.93 \pm 4.7	—
		>5yr	M	8.1 \pm 1.18	—	68.4 \pm 6.0	—	22.0 \pm 4.08	—	3.7 \pm 1.34	—	1.2 \pm 1.4	—	5.1 \pm 2.6	—
11	Winter	NS	Both	8.57 \pm 0.25	—	44 \pm 8.9	—	50.78 \pm 7.8	—	3.7 \pm 2.1	—	0	—	2.7 \pm 0.6	—
	Spring	NS	Both	8.47 \pm 1.85	—	43 \pm 9	—	53.3 \pm 8.1	—	2.0 \pm 1.7	—	0	—	2.0 \pm 1.7	—
	Summer	NS	Both	6.43 \pm 0.89	—	50.2 \pm 12.8	—	37.8 \pm 11.3	—	8.6 \pm 3.0	—	0.3 \pm 0.5	—	4.5 \pm 2.6	—
	Fall	NS	Both	6.2 \pm 0.64	—	48.2 \pm 4.6	—	40.8 \pm 4.9	—	8.0 \pm 4.2	—	0.2 \pm 0.4	—	2.6 \pm 0.9	—
7	Ranched	NS	NS	6.6 \pm 1.2	—	—	—	—	—	—	—	—	—	—	—
	Park 1	Juvenile	NS	9.1 \pm 1.5	—	—	—	—	—	—	—	—	—	—	—
	Park 1	Adult	NS	7.9 \pm 1.9	—	—	—	—	—	—	—	—	—	—	—
	Park 2	Juvenile	NS	11.3 \pm 1.8	—	—	—	—	—	—	—	—	—	—	—
	Park 2	Adult	NS	8.4 \pm 2.2	—	—	—	—	—	—	—	—	—	—	—
2		NS	NS	—	—	44.2 \pm 3.6	—	46.1 \pm 3.1	—	10.8 \pm 1.7	—	0	—	2.6 \pm 0.6	—
15		1–2yr	M/F	7.72 \pm 1.7	3.4–18.4	43.3	—	51.2	—	3.6	—	0.27	—	0.75	—
19	Ranched	All	M/F	10.56 \pm 3.62	—	43.8 \pm 15.46	—	44.0 \pm 16.38	—	6.6 \pm 5.31	—	0	—	0.65 \pm 0.75	—
	Ranched	Calf	M/F	9.64 \pm 2.99	—	36.2	—	53.2	—	5.2	—	0	—	4.8	—
		(<185 kg)													
	Ranched	Adult	M/F	11.48 \pm 3.78	—	51.4	—	34.8	—	9.1	—	0	—	4.5	—
	(>185 kg)														
	Free-ranging	All	M/F	—	—	41.75 \pm 11.47	—	50.8 \pm 11.66	—	4.1 \pm 2.67	—	0	—	3.25 \pm 3.91	—
21		<6mo	M/F	8.45 \pm 0.94	—	29	—	65.7	—	0.95	—	0	—	4	—
		7–23mo	M/F	9.38 \pm 0.79	—	42	—	49.4	—	3.4	—	0.4	—	4.3	—
		>24mo	M/F	10.59 \pm 0.52	—	49	—	43.9	—	3.7	—	0.3	—	3	—
22		<1yr	M/F	4.9 \pm 0.42	1.2–11.8	29.91 \pm 3.56	13–49	54.55 \pm 4.14	34–81	4.18 \pm 1.31	0–11	0.18 \pm 0.12	0–1	1.73 \pm 0.47	0–5
		Adult	M	4.22 \pm 0.35	1.5–14.1	36.53 \pm 5.33	0–78	44.44 \pm 4.45	11–86	3.56 \pm 0.8	0–10	0	0	3.31 \pm 0.71	0–8
		Adult	F	4.43 \pm 0.22	1.0–11.13	31.54 \pm 2.61	12–76	48.61 \pm 3.83	8–86	2.96 \pm 0.49	0–11	0.14 \pm 0.07	0–1	1.71 \pm 0.34	0–6

NS, not specified.

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Hematology of Reindeer

BRIDGET C. GARNER

Properties of Blood Cells

- Erythrocytes
- Leukocytes
 - Neutrophils
 - Lymphocytes
 - Eosinophils
 - Monocytes
 - Basophils
- Platelets

Reference Intervals

- Effects of Age
- Effects of Gender
- Effects of Reproductive Status and Seasonal Changes
- Effects of Blood Sampling

Acronyms and Abbreviations

CBC, complete blood count; Hgb, hemoglobin; LGL, large granular lymphocyte; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PCV, packed cell volume; RBC, red blood cell; WBC, white blood cell.

Routine hematology is not often performed in reindeer (*Rangifer tarandus*; order Artiodactyla, family Cervidae), but reindeer hematology has received more attention than usual in recent years. A new hemomycoplasma organism that may cause anemia in reindeer was recently identified.⁷ An unrelated reindeer was diagnosed with a potentially new *Theileria* sp. most similar to *Theileria cervi* type F (B.C. Garner, in preparation). With the introduction of these previously unidentified infectious agents it becomes increasingly important to know what is normal for a healthy reindeer. Several factors can affect the hematologic parameters of normal reindeer. Such variables include the animal's age, gender, and reproductive status, as well as the stress of blood collection. These effects on the normal components of the reindeer complete blood count (CBC) are discussed in this chapter.

PROPERTIES OF BLOOD CELLS

Erythrocytes

The erythrocytes of reindeer are discoid with an average diameter of 5.5 μm .³ A standard peripheral blood smear from a healthy animal shows mild anisocytosis, but polychromasia is rare. Central pallor, although present,

is often indistinct. Reindeer erythrocytes do not sickle *in vitro* when exposed to oxygen like erythrocytes from others within the Cervidae family (discussed in Chapter 116), and rouleaux formation is uncommon.³

Leukocytes

The morphology, cytochemistry, and ultrastructure of reindeer leukocytes have been extensively studied (Figs. 119.1–119.6).^{2,3} On a stained peripheral blood smear, the granulocytes and mononuclear cells of reindeer can be readily identified.

Neutrophils

Neutrophils comprise nearly half of all leukocytes (Table 119.1).^{1,3} They are typically 18–25 μm in diameter with pale eosinophilic cytoplasm. Reindeer neutrophils may occasionally exhibit a low number of basophilic cytoplasmic granules. The nuclei resemble those of other species, but they may be more segmented with up to seven lobes.²

Lymphocytes

Lymphocytes are typically the second most abundant leukocyte in healthy reindeer. Their diameters range

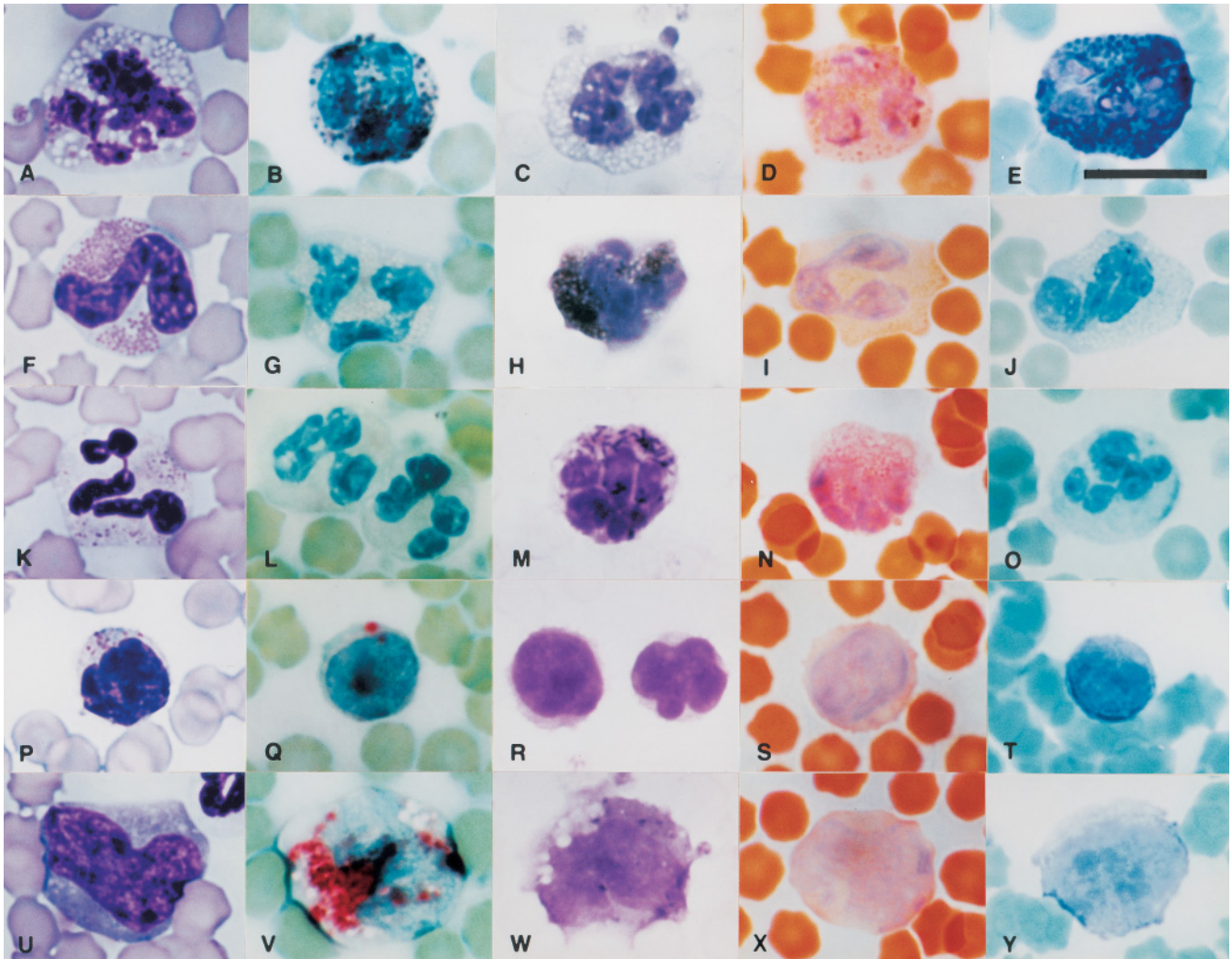


FIGURE 119.1 Cytochemical staining of reindeer leukocytes. (A) Gray basophil, Romanowsky's stain. (B) Basophil, alpha-naphthyl butyrate esterase (α -NBE). (C) Basophil, Sudan black B (SBB). (D) Basophil, chloroacetate esterase (CAE). (E) Basophil, alkaline phosphatase (ALP). (F) Eosinophil, Romanowsky's stain. (G) Eosinophil, α -NBE. (H) Eosinophil, SBB. (I) Eosinophil, CAE. (J) Eosinophil, ALP. (K) Neutrophil, Romanowsky's stain. (L) Neutrophil, α -NBE. (M) Neutrophil, SBB. (N) Neutrophil, CAE. (O) Neutrophil, ALP. (P) Lymphocyte, Romanowsky's stain. (Q) Lymphocyte, α -NBE. (R) Lymphocyte, SBB. (S) Lymphocyte, CAE. (T) Lymphocyte, ALP. (U) Monocyte, Romanowsky's stain. (V) Monocyte, α -NBE. (W) Monocyte, SBB. (X) Monocyte, CAE. (Y) Monocyte, ALP. Scale bar = 10 μ m. (Reproduced from Henkel KA, Swenson CL, Richardson B, et al. Morphology, cytochemical staining and ultrastructural characteristics of reindeer (*Rangifer tarandus*) leukocytes. *Vet Clin Pathol* 1999;28:8-15, with permission.)

from 15 to 35 μ m, and they may be round, oval, or angular in shape, with variable nuclear to cytoplasmic ratios. The cytoplasm, which is usually lightly basophilic, may contain a few fine azurophilic granules. This cellular morphology is consistent with that of large granular lymphocytes (LGLs).

Eosinophils

Eosinophils are slightly larger than neutrophils at 25–30 μ m in diameter. The red-orange cytoplasmic granules are typically small and round, with an electron-dense appearance via electron microscopy.² The granules frequently exhibit polar or bipolar clustering as they form aggregates on one or both sides of the nucleus.²

Monocytes

Monocytes have reniform to oval-shaped nuclei with pale, blue-gray cytoplasm on peripheral blood smears. Occasionally, there are clear cytoplasmic vacuoles and a small quantity of red-to-pink cytoplasmic granulation.²

Basophils

The reindeer is somewhat unique in that it has two distinct basophil populations (Fig. 119.7). The first has the typical appearance noted within most other mammals. These are characterized by numerous, pale to dark basophilic granules throughout the entire

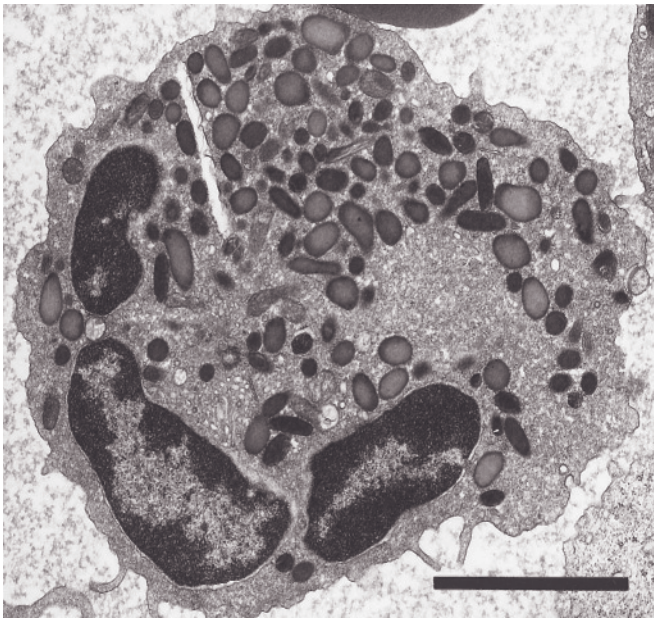


FIGURE 119.2 Electron micrograph, reindeer neutrophil. Scale bar = 2 μ m. (Reproduced from Henkel KA, Swenson CL, Richardson B, et al. Morphology, cytochemical staining and ultrastructural characteristics of reindeer (*Rangifer tarandus*) leukocytes. *Vet Clin Pathol* 1999;28:8-15, with permission.)

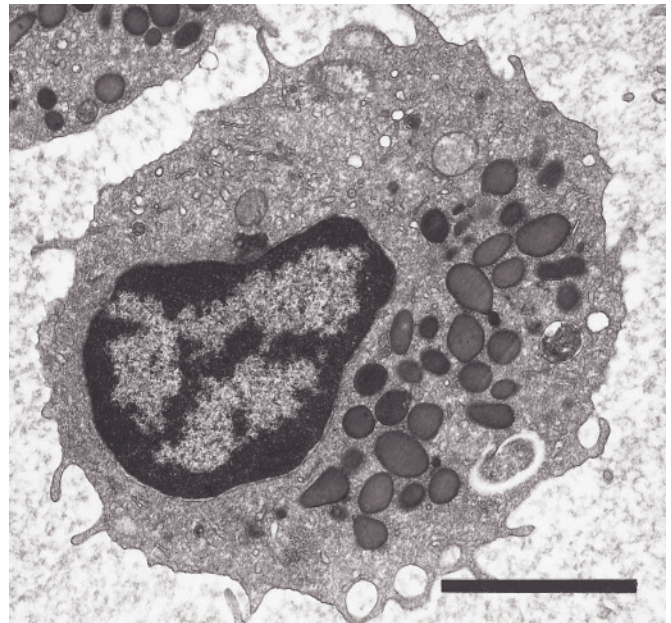


FIGURE 119.4 Electron micrograph, reindeer eosinophil. Scale bar = 2 μ m. (Reproduced from Henkel KA, Swenson CL, Richardson B, et al. Morphology, cytochemical staining and ultrastructural characteristics of reindeer (*Rangifer tarandus*) leukocytes. *Vet Clin Pathol* 1999;28:8-15, with permission.)

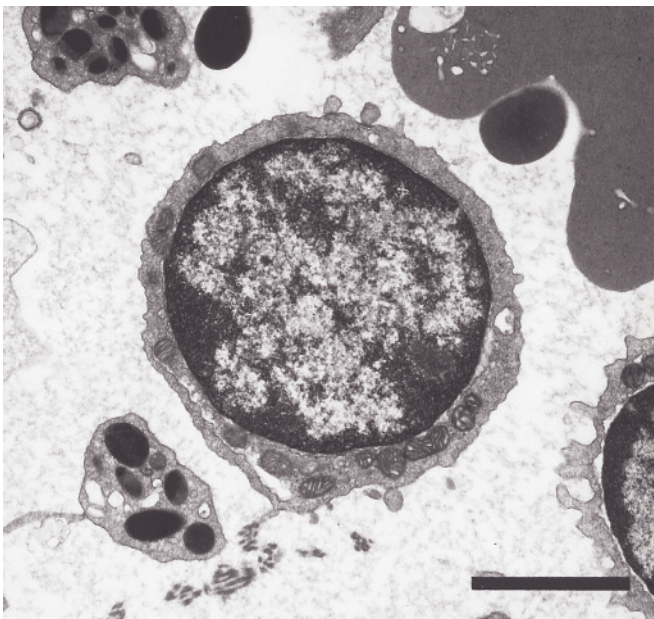


FIGURE 119.3 Electron micrograph, reindeer lymphocyte. Scale bar = 2 μ m. (Reproduced from Henkel KA, Swenson CL, Richardson B, et al. Morphology, cytochemical staining and ultrastructural characteristics of reindeer (*Rangifer tarandus*) leukocytes. *Vet Clin Pathol* 1999;28:8-15, with permission.)

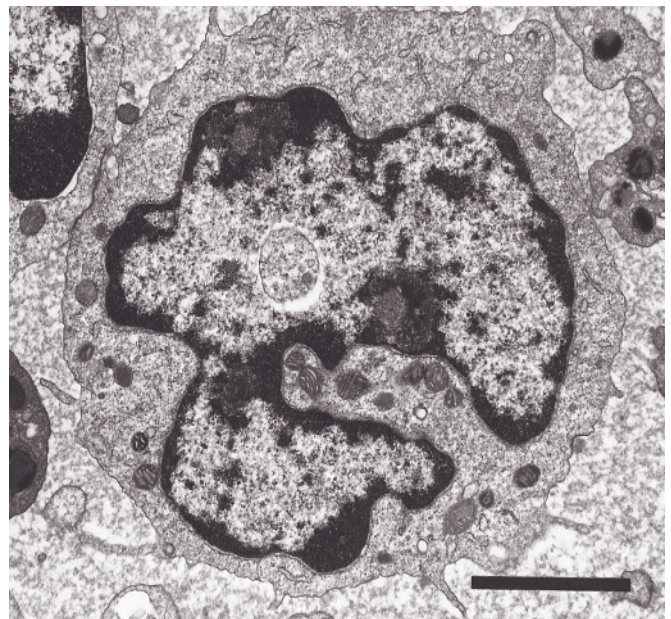


FIGURE 119.5 Electron micrograph, reindeer monocyte. Scale bar = 2 μ m. (Reproduced from Henkel KA, Swenson CL, Richardson B, et al. Morphology, cytochemical staining and ultrastructural characteristics of reindeer (*Rangifer tarandus*) leukocytes. *Vet Clin Pathol* 1999;28:8-15, with permission.)

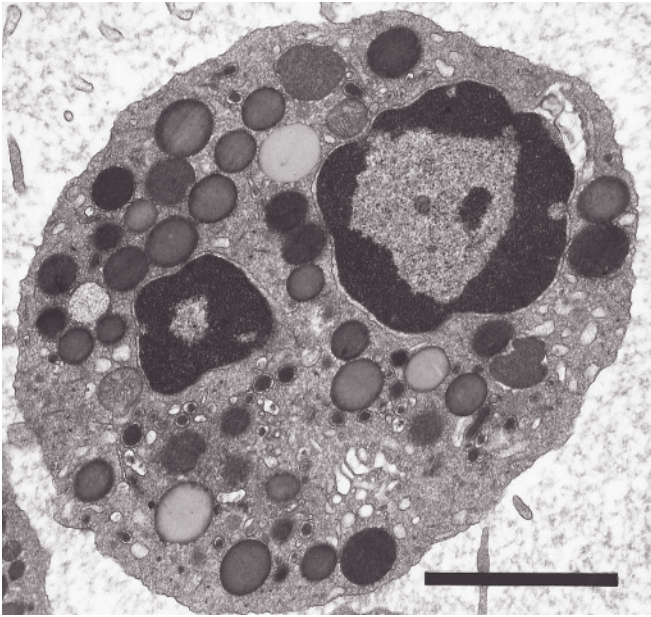


FIGURE 119.6 Electron micrograph, reindeer basophil. Scale bar = 2 μ m. (Reproduced from Henkel KA, Swenson CL, Richardson B, et al. Morphology, cytochemical staining and ultrastructural characteristics of reindeer (*Rangifer tarandus*) leukocytes. Vet Clin Pathol 1999;28:8–15, with permission.)

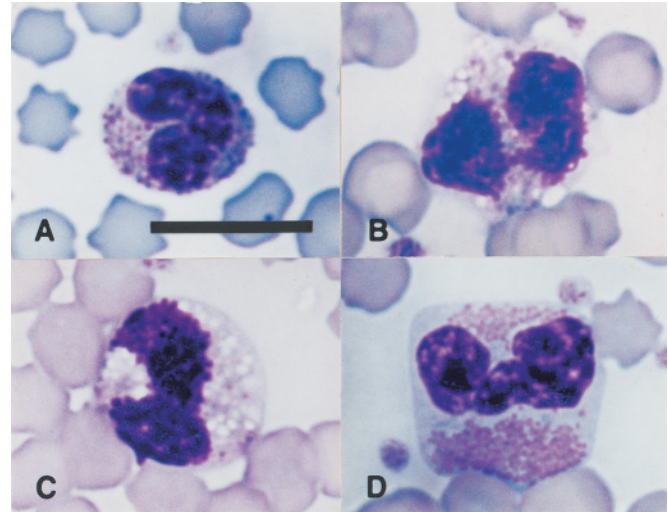


FIGURE 119.7 Comparison of a dark basophil (A) with gray basophils (B, C) and an eosinophil (D) from reindeer blood. The dark basophil granules are similar to basophil granules in other ruminants. Romanowsky's stain; scale bar = 10 μ m. (Reproduced from Henkel KA, Swenson CL, Richardson B, et al. Morphology, cytochemical staining and ultrastructural characteristics of reindeer (*Rangifer tarandus*) leukocytes. Vet Clin Pathol 1999;28:8–15, with permission.)

TABLE 119.1 Complete Blood Count Data from Healthy Adult Male and Female Reindeer Housed in a Zoological Park

Analyte	Adult Male (n = 14)		Adult Female (n = 16)	
	Mean \pm S.D.	Range	Mean \pm S.D.	Range
WBC ($\times 10^3/\mu$ L)	5.2 \pm 2.0	3.1–12.0	7.0 \pm 2.1	3.0–11.7
Neutrophils ($\times 10^3/\mu$ L)	2.6 \pm 1.0	1.4–4.6	3.6 \pm 1.2	1.8–6.0
Lymphocytes ($\times 10^3/\mu$ L)	1.6 \pm 1.0	0.7–5.3	2.2 \pm 1.0	0.6–6.1
Monocytes ($\times 10^3/\mu$ L)	0.1	0.0–0.4	0.1	0.0–1.0
Eosinophils ($\times 10^3/\mu$ L)	0.8 \pm 0.7	0.0–2.9	0.9 \pm 0.6	0.0–2.5
Basophils ($\times 10^3/\mu$ L)	0.2	0.0–0.9	0.3	0.0–0.9
RBC ($\times 10^6/\mu$ L)	9.3 \pm 1.17	7.9–12.3	9.6 \pm 1.33	7.7–11.96
Hgb (g/dL)	16.3 \pm 2.2	13.9–21.7	18.1 \pm 2.1	13.7–22.4
PCV (%)	42 \pm 5	34–54	47 \pm 6	34–56
MCV (fL)	42.2 \pm 2.6	41.6–50.8	48.3 \pm 2.8	44.5–54.9
MCH (pg)	17.3 \pm 1.0	15.7–19.1	18.6 \pm 1.1	16.9–20.9
MCHC (g/dL)	38.4 \pm 1.7	35.7–43.5	38.6 \pm 1.3	35.9–41.4
Fibrinogen (g/dL)	0.26 \pm 0.07	0.2–0.42	0.26 \pm 0.09	0.14–0.46
Platelets ($\times 10^3/\mu$ L)	323 \pm 127	140–675	328 \pm 143	175–613

Adapted from Catley A, Kock RA, Hart MG, et al. Haematology of clinically normal and sick captive reindeer *Rangifer tarandus*. Vet Rec 1990;126:239–241.

cytoplasm. The second population is cytologically similar to “gray” basophils seen in limited other species. These cells have variable numbers of clear cytoplasmic granules (or vacuoles) that are less than 1.0 μ m in diameter interspersed with variable numbers of intensely basophilic, perinuclear granules of similar size. The perinuclear granules may obscure a portion of the nucleus and make it appear tattered or deformed. Both basophil populations can be identified within the same

blood smear, and the relative proportions of each can vary between individuals.²

Platelets

The platelets of reindeer are relatively small (Fig. 119.7), and the reference interval for platelet quantity is wide (Tables 119.1, 119.2).

TABLE 119.2 Hematological Reference Values for Healthy Reindeer of Different Ages

Analyte	Newborn (0–14 days) n = 11		Juveniles (1–18 months) n = 22		Adults n = 30	
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
WBC ($\times 10^3/\mu\text{L}$)	4.36 ± 0.22	1.60–8.30	6.75 ± 2.19	2.70–12.90	6.33 ± 2.12	2.80–12.00
Neutrophils ($\times 10^3/\mu\text{L}$)	2.48 ± 1.48	0.61–4.73	3.10 ± 1.21	1.49–6.97	3.19 ± 1.25	1.29–6.59
Lymphocytes ($\times 10^3/\mu\text{L}$)	1.74 ± 0.66	0.94–2.77	2.46 ± 0.95	0.60–4.50	2.01 ± 0.93	0.69–6.08
Monocytes ($\times 10^3/\mu\text{L}$)	0.12	0.0–0.3	0.16	0.0–0.65	0.10	0.0–1.0
Eosinophils ($\times 10^3/\mu\text{L}$)	0.26	0.0–2.1	0.67	0.0–2.0	0.79	0.0–2.88
Basophils ($\times 10^3/\mu\text{L}$)	0.13	0.0–0.8	0.28	0.0–0.8	0.24	0.0–1.05
RBC ($\times 10^6/\mu\text{L}$)	8.16 ± 0.93	6.38–9.89	10.60 ± 1.2	8.16–13.14	9.29 ± 1.27	6.94–12.58
Hgb (g/dL)	13.67 ± 1.22	11.50–15.90	18.58 ± 1.68	14.60–21.90	17.2 ± 2.2	13.7–22.4
PCV (%)	39 ± 4	31–43	49 ± 5	40–56	44 ± 6	34–56
MCV (fL)	47.4 ± 4.0	37.4–51.3	46.6 ± 5.5	38.0–63.7	47.8 ± 3.4	41.6–55.7
MCH (pg)	16.8 ± 1.4	14.0–18.9	17.6 ± 1.8	14.4–22.9	18.5 ± 1.3	15.7–21.4
MCHC (g/dL)	35.5 ± 1.5	32.6–37.3	37.7 ± 1.2	34.7–39.8	38.1 ± 1.0	35.7–39.8
Fibrinogen (g/dL)	0.33 ± 0.11	0.12–0.49	0.25 ± 0.08	0.12–0.4	0.27 ± 0.13	0.14–0.46
Platelets ($\times 10^3/\mu\text{L}$)	514 ± 237	149–975	334 ± 103	124–472	336 ± 118	140–675
Reticulocytes (%)	0.1	0.0–28.0	0.04	0.0–3.9	0.02	0.0–1.1

Adapted from Catley A, Kock RA, Hart MG, et al. Haematology of clinically normal and sick captive reindeer *Rangifer tarandus*. Vet Rec 1990;126:239–241.

REFERENCE INTERVALS

Effects of Age

Age has several effects on hematologic values (Table 119.2). Compared to juveniles and adults, newborns have lower hemoglobin (Hgb) levels, red blood cell (RBC) counts, and packed cell volume (PCV).^{1,8} Mean corpuscular hemoglobin concentration (MCHC), white blood cell (WBC) count, and eosinophil counts are also often lower in newborns compared to juveniles. In contrast reticulocyte and platelet counts are higher in newborns. Marked red cell anisocytosis and poikilocytosis can be found in blood smears from healthy newborn reindeer, but only mild anisocytosis is expected in normal adults. Basophilic stippling and a small number of nucleated RBCs may also be identified in the neonates.¹

Juveniles (up to 18 months old) have the highest Hgb and PCV values. Lymphocyte and RBC counts are also higher in juveniles than adults. Reindeer hematologic values stabilize at approximately 5 months of age; therefore, adult reference intervals may be useful when interpreting data from animals 5 months or older if juvenile reference intervals are not available.^{3,8}

Effects of Gender

Adult females living in zoological parks have higher Hgb, PCV, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and WBC counts than adult males (Table 119.1).⁸ These values are higher (and may peak) in females during the autumn months.⁸ Published CBC data from two adult female and two adult male reindeer living on a Michigan farm fell within these previously established reference intervals, but the disparities between the males and females were less drastic.² Significant differences between newborn

and juvenile males and females are not reported as gender-related variations in hematologic parameters typically become more significant with sexual maturity and adulthood.¹

Effects of Reproductive Status and Seasonal Changes

The Hgb concentration of pregnant females decreases by approximately 3g/dL during winter and decreases further following parturition.⁸ Hemoglobin increases sharply during the summer toward its peak in autumn. Plasma total protein concentrations decline during winter and are at their lowest in May and their highest in October.⁵ Similar fluctuations due to changing seasons and reproductive status are reported in other hematologic parameters.⁸

Effects of Blood Sampling

A stress response to various handling conditions has been repeatedly demonstrated in reindeer.^{4,6} As reported in other species, reindeer stressed during venipuncture may demonstrate lymphopenia and eosinopenia, as well as moderate increases in PCV, Hgb and RBC count.⁴ Another study showed five- to six-fold increases in plasma cortisol and significant increases in plasma noradrenaline during manual blood sampling of eight mature reindeer.⁶

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Hematology of Elephants

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Blood and Bone Marrow Collection
Erythrocytes
Leukocytes
Platelets and Coagulation
Reference Intervals

Hematologic Disease
Tuberculosis
Elephant Endotheliotropic Herpesvirus
Blood Parasites
Summary

Acronyms and Abbreviations

ANAE, alpha naphthyl acetate esterase; ANBE, alpha naphthyl butyrate esterase; CAE, chloroacetate esterase; CO, carbon monoxide; DCF, dichlorofluorescein; DCFH, dichlorofluorescein; ESR, erythrocyte sedimentation rate; Hgb, hemoglobin; LAP, leukocyte alkaline phosphatase; Mgb, myoglobin; MCV, mean corpuscular volume; PAF, platelet activating factor; PCV, packed cell volume; RBC, red blood cell; TEM, transmission electron microscopy.

Hematology values are available for two main species of elephants. The Asian elephant, *Elephas maximus*, has lived in human society since the time of ancient India, while the African elephant, *Loxodonta africana*, has not been as closely associated with human culture and is more difficult to manage in captivity.

Accurate interpretation of CBC results for elephants depends on the ability to correctly identify leukocyte morphology and cell types.

BLOOD AND BONE MARROW COLLECTION

A blood volume of over 100L was measured in an Asian elephant weighing 3216kg.²⁹ This represents a blood volume of approximately 3.5% body weight. Blood can be collected from several of the subcutaneous auricular veins located on the medial (caudal) surface of the ear. Many elephants are trained to allow access to the ear for routine venipuncture while standing and are not physically restrained or sedated. Alternatively, the elephant can be trained to lie down on the ground. For access, the ear must be pulled forward and away from the skin of the neck by the handler or assistant. The collector stands caudal to the ear where the caudal surface can be examined and palpated. The skin of the

ear should be wiped clean of loose debris; the selected site should be cleaned and prepared for standard aseptic venipuncture. Care must be taken to distinguish the soft fluctuant veins from the firm, pulsatile arteries of the ear. Collection from one of the auricular arteries produces subcutaneous hematomas that can occasionally leave disfiguring fibrous scars in the ear. In general, the ear veins are subject to large fluctuations in size due to temperature and blood pressure dependent factors. The veins of the ear can be very difficult to visualize or palpate if attempting to collect blood when the air temperature is below 21°C (70°F). Placing mild heat to the medial surface of the ear for several minutes with a warmed moist towel or forced heated air can help dilate the veins; however, care must be taken not to cause thermal injury to the skin of the ear. Alternatively, auricular vasodilatation can be induced by vigorously exercising the elephant prior to blood collection or by allowing the animals to stand outside in the sun.

Due to the thickness of the skin, there are few other venipuncture sites available for blood collection. The lateral (anterior) surface of the ear may have visible veins. The medial saphenous veins located on the inner surfaces of the hind legs are accessible for venipuncture. These large vessels are usually visible and not as subject to variations in size due to ambient temperatures. To provide safe access to this site, the animal must be

trained to allow manipulation of the vein without moving or kicking. Blood can also be obtained from the ventral tail vein as described in bovine species. Any collected blood should be placed in EDTA anticoagulant tubes and refrigerated until processed.

Although elephant bone marrow has not been well described to date, a technique for its collection has been described but is rarely performed.⁴ Bone marrow is absent from the ribs and long bones of elephants, but is present in the dorsal spinous processes of the first lumbar vertebra.²²

ERYTHROCYTES

Elephants have the largest erythrocytes of terrestrial mammals, measuring an average of 125 fL. Early reports in the 1950s report mean cell volumes of up to 168 fL.⁷ The packed cell volume (PCV) averages around 35% and ranges from 25% to 45%, similar to domestic livestock.^{23,30,31} The red blood cell (RBC) count is lower at an average of $2.9 \times 10^6/\mu\text{L}$ and ranges from $1.7 \times 10^6/\mu\text{L}$ to $5.0 \times 10^6/\mu\text{L}$ in nonpregnant adults.^{23,31} Pregnant females have been reported to have a lower average RBC count of $1.8 \times 10^6/\mu\text{L}$ and a PCV of 30% with increased erythrocyte sedimentation, likely due to volume expansion and hormonal effects during fetal development.²³ These numbers are remarkably similar to one of the elephant's closest evolutionary cousins, the manatee.¹³

The degree of anisocytosis is similar to that observed in the manatee and in cattle.¹³ Low numbers of Howell-Jolly bodies may be found if blood films are scanned at low power. Elephant RBCs have a high resistance to osmotic lysis, which may be related to the large surface/volume ratio of the biconcave disc.³² The lifespan of the elephant RBC has not been reported at this time.

Reticulocytes are typically absent from the blood of apparently healthy African and Asian elephants.^{1,14,15} Nucleated erythrocytes are very rarely recognized in healthy animals and should be considered an abnormal finding. Reticulocytes may be observed in the blood of anemic elephants.

The erythrocyte sedimentation rate (ESR) has revealed dramatically different results in different authors' hands: 29 mm/hr,³⁹ 21–62 mm/hr (African) and 36–64 mm/hr (Asian),⁵ and a mean of 120 mm/hr with a range of 64–148 mm/hr.³¹ This is quite rapid in elephants, either due to erythrocyte size, the ability of elephant RBC to form rouleaux, interaction with the protein matrix, or most likely, a combination thereof. Though this analysis is now rarely performed clinically, this may be important when considering sample handling in the laboratory, or post-mortem collection. Cells will settle very rapidly and accurate quantification requires continuous rocking.

It has been reported that an increased PCV due to splenic contraction and stress is unlikely as the splenic capsule has been reported to be fibrous similar to a cow.²² To date, increased PCV due to stress and splenic contraction has not been documented in the elephant.

Elephant hemoglobin (Hgb)^{19,28} and myoglobin (Mgb)¹⁸ studies reveal some structural changes at the protein level. Elephant Hgb has been sequenced and found to have alpha-chains that differ by 24 exchanges and beta-chains by 27 exchanges from the human sequence. There are only five phosphate binding sites, similar to that of the llama. The elephant has a low P_{O_250} and reduced interaction with 2,3-bisphosphoglycerate in comparison to humans.¹⁹ Elephant Mgb has glutamine in the distal, heme-binding position instead of histidine. Elephant Mgb has a carbon monoxide (CO) affinity approximately six times higher than that of human or sperm whale Mgb,¹⁸ and reacts with nitric oxide 500–1000 times faster than does human Mgb.²⁸ It is postulated that new interactions resulting from the conformational change accompanying ligand binding may be responsible for the increased CO binding.¹⁸

LEUKOCYTES

A combination of Wright-Giemsa, cytochemical stains, and transmission electron microscopy (TEM) demonstrates that all elephant leukocyte types other than monocytes are similar to other mammalian leukocytes, as shown in Figure 120.1. Monocytes with round to pleomorphic to distinctly bilobed nuclei are found in elephants.

Asian and African elephants have heterophils rather than neutrophils. Using light microscopy under 400 \times power, Wright-Giemsa stained elephant heterophils measure 12–16 μm in diameter. Heterophil nuclei contain dark, coarsely clumped chromatin and are moderately lobulated with an average of four to five segments, similar to equine species. The large quantity of clear to light blue cytoplasm generally contains more than 30 fine, variably sized, round, oval or rod shaped pink granules. The fine granules in some heterophils stain weakly to moderately positive with peroxidase while other heterophils are negative for peroxidase stain. Individual variability between elephants is noted. Heterophils also stain slightly to moderately positively for leukocyte alkaline phosphatase (LAP) and chloroacetate esterase (CAE). Heterophils are negative for both alpha naphthyl acetate esterase (ANAE) and alpha naphthyl butyrate esterase (ANBE). Heterophil granules are round and are 400–700 nm in diameter or can be elongated, oblong structures up to 670 nm \times 600 nm, as seen using transmission electron microscopy (TEM). There are two types of distinct granules present, with primary granules being larger and electrolucent. The secondary granules contain a distinct band of electrofluorescence. Heterophils contain few mitochondria and varying numbers of tubulovesicular structures as well as numerous vacuoles. Vacuolation is observed to increase with toxic change in the heterophil line.

Oxidative metabolic burst activity of Asian elephant heterophils and Holstein cattle neutrophils has been compared indirectly using the oxidation of non-fluorescent intracellular dichlorofluorescein (DCFH) to fluorescent dichlorofluorescein (DCF).³³ Phorbol myristate

	WG	Peroxidase	LAP & Peroxidase	CAE	ANAE	TEM
Heterophil						
Lymphocyte						
Bilobed Monocyte						
Round Monocyte						
Eosinophil						
Basophil						
Platelets						

Abbreviations: WG- Wright Giemsa, LAP- Leukocyte Alkaline Phosphatase, CAE- Chloroacetate Esterase, ANBE- Alpha Naphthyl Butyrate Esterase, TEM- Transmission Electron Microscopy.

Transmission electron microscopy photos courtesy of Dr. Nicole Stacy. Photo of basophil courtesy of Dr. Eric Morrisette. CAE and ANBE staining photos courtesy of Dr. Rose Raskin; other staining photos courtesy of Dr. Julia Blue and Dr. Kendal Harr..

FIGURE 120.1 Representative images of the staining and ultrastructural properties of elephant leukocytes and platelets. Abbreviations: WG, Wright-Giemsa; LAP, leukocyte alkaline phosphatase; CAE, chloroacetate esterase; ANBE, alpha naphthyl butyrate esterase; TEM, transmission electron microscopy. (Transmission electron microscopy photos courtesy of Dr. Nicole Stacy. Photo of basophil courtesy of Dr. Eric Morrisette. CAE and ANBE staining photos courtesy of Dr. Rose Raskin; other staining photos courtesy of Dr. Julia Blue and Dr. Kendal Harr.)

acetate was used to activate the cells. Elephant heterophils and bovine neutrophils oxidized intracellular DCFH to DCF in a similar manner. A wide range of mean channel fluorescence intensity (1803–7393) was observed among individual elephants which was similar to that observed in the bovine neutrophils.

Elephant lymphocytes evaluated with Wright-Giemsa stain on light microscopy are variable in size, ranging from small to large. Medium sized lymphocytes predominate in most animals. In some apparently healthy animals there is a predominance of large lymphocytes. Nuclei are round to oval and occasionally

cleaved. Chromatin is diffuse in larger cells but in smaller cells is denser with 1–3 chromocenters. There is a scant rim of pale basophilic cytoplasm which rarely contains large purple granules approximately 2µm in diameter. Lymphocytes do not stain with peroxidase, CAE, or alkaline phosphatase stains and only occasionally show focal staining with ANBE stain (Fig. 120.1). Ultrastructurally, lymphocytes are agranular or contain a few round to oval granules ranging from 100 to 200 nm in diameter with homogeneous or granular matrices of varying electron density, and occasional myelin figures. Lymphocytes contain many mitochondria and varying numbers of tubulovesicular structures.

Discrepancy in the literature regarding a unique bilobed leukocyte and differentiation of lymphocytes and monocytes^{1,11,22,30,31} has resulted in highly variable and imprecise results, likely due to misidentification of this cell type. The bilobed cells are identical in cytochemical staining to the traditional monocyte. Monocytes are typically positive for peroxidase while lymphocytes are typically negative for peroxidase. The presence of small granules and phagocytic vacuoles with rare mitochondria on electron microscopy supports identification of both the bilobed cell and round nuclear cell types as monocytes (see below).

Monocytes of three distinct morphologies are present in elephant blood. The monocyte typical of most domestic mammals, with a rounded, polymorphic nucleus with clumped chromatin and moderate quantities of vacuolated basophilic cytoplasm when stained with Wright-Giemsa, is also present in elephants in low numbers. The overall cell size is >20µm. The second type of monocyte is bilobed, with an overall cell size approximately 16–18µm diameter. The nuclei are horseshoe-shaped to bilobed, and the cytoplasm is lightly basophilic using a Wright-Giemsa stain. The cytoplasm has an irregular border with cytoplasmic vacuoles occasionally present. It is also important to note that there are monocytes that, when spread on a glass slide, appear to have a round to oval to slightly indented nucleus similar to the lymphocyte population. The chromatin pattern, however, is coarse to clumped and vacuoles are typically present in the relatively small amount of cytoplasm. When stained with peroxidase these cells, especially the vacuoles, are moderately peroxidase positive which clearly differentiates them from nearby lymphocytes (Fig. 120.1).

All monocytes have 1–2 positive granules when stained with peroxidase, indicating a phagocytic function. This weak staining was also found in a study of the bilobed leukocyte in African elephants.^{1,38} When stained with peroxidase, monocytes demonstrate weak peroxidase activity in rats,³⁷ humans, and other species.^{3,16,17,24} The peroxidase activity in the bilobed leukocyte provides further evidence that these are not likely to be lymphocytes, which lack myeloperoxidase granules.

The monocytes are usually negative for CAE; however, weak positive areas that may be vacuoles, are present in a few monocytes. Chloroacetate esterase typically stains positively in neutrophils and mast cells. Monocytes of other species have been noted to be

weakly reactive with this stain.¹⁶ In contrast, lymphocytes, plasma cells, eosinophils and blast stages of erythrocytes will all stain completely negative.

All of these monocytes are negative for LAP, ANAE, and ANBE. Alkaline phosphatase may be found in mature neutrophils/heterophils in some species, thus supporting evidence that this unique cell type is not of the neutrophil line.

Ultrastructurally, round monocytes contain small numbers of round granules, 200–300nm in diameter, with granular matrices of variable electron density. Mitochondria are numerous, and tubulovesicular structures are present in varying numbers. There are rare, diffuse granules present along with vacuoles surrounded by an indistinct membrane. Ultrastructurally, bilobed monocytes are also similar to the round monocyte type. They contain small numbers of round granules, varying from 100 to 300nm in diameter, with granular matrices of variable electron density surrounded by an indistinct membrane. Mitochondria and tubulovesicular structures are present in varying numbers. The bilobed cell type has a larger nucleus relative to cytoplasm volume than the traditional monocytes encountered. Additionally, the bilobed monocyte has a slightly smaller cell size than the more typical monocyte.

Eosinophil cell size varies from 14µm to 18µm in diameter. Nuclei are trilobed, bilobed, or band-shaped, and have coarsely clumped chromatin that is less condensed than neutrophil chromatin. Nuclei are 6–7µm × 11–12µm in size. Eosinophils have pale basophilic cytoplasm, with a staining intensity intermediate between neutrophils and mononuclear cells. The eosinophil granules are red-orange, round, less than 1µm in diameter, and distinctly separate. On peroxidase, numerous, strongly positive, ovoid granules are present. When stained for CAE using a purple chromagen, eosinophils have a diffuse, pale, orange background with refractile granules suggesting a negative reaction. Eosinophils are negative for alkaline phosphatase; however, both ANBE and ANAE produce a strong reaction. Ultrastructurally, eosinophil granules are round to oval and vary from 300 to 600nm in size. The granular matrix is moderately electron dense and homogeneous, with a tendency towards greater electron density near the periphery. Few mitochondria and varying numbers of tubulovesicular structures are present.

Basophils measure 14–18µm in diameter. The cytoplasm is clear to basophilic and contains lavender to magenta staining punctate granules. Nuclei are bilobed to band shaped with clumped chromatin that is not as coarse as heterophils. Because there are generally low numbers in patient samples, these cells are only confirmed in peroxidase stain. Basophils are negative when stained with peroxidase, which is consistent with findings in rats and humans.² Further study is required. One study of 23 elephants found basophils in only two of the individual animals.³⁹ Reported reference intervals indicate a mean value of less than 40 basophils/µL in African elephants and 160/µL in Asian elephants.⁵ A

TABLE 120.1 Summary of Results of Cytochemical Staining for Elephant Leukocytes and Platelets

	Heterophil	Lymphocyte	Round Monocyte	Bilobed Monocyte	Eosinophil	Basophil	Platelet
Peroxidase	+	–	+	+	+	–	–
Chloroacetate esterase	+	±	±	±	–	±	–
Leukocyte alkaline phosphatase	+	–	–	–	–	NA	NA
Alpha naphthyl acetate esterase	–	focal +	–	–	+	NA	NA
Alpha naphthyl butyrate esterase	–	–	–	–	+	NA	–

Abbreviations: –, negative; +, positive; ±, weak positive; NA, not available

summary of cytochemical reactions for leukocytes and platelets is shown in Table 120.1.

PLATELETS AND COAGULATION

In observations by chapter authors, using a CellDyn 3500, platelets were found to range from $342 \times 10^3/\mu\text{L}$ to $719 \times 10^3/\mu\text{L}$ in Asian elephants, with a relatively small mean platelet volume. This is similar to the literature which reports $540 \times 10^3/\mu\text{L}$ to $637 \times 10^3/\mu\text{L}$ in Asian elephants and $294 \times 10^3/\mu\text{L}$ to $455 \times 10^3/\mu\text{L}$ in African elephants.⁵ It should be noted that the reported reference intervals from the literature are based on post-mortem samples and are likely lower than would be found in well-handled samples from live animals.

A detailed study of blood platelets and their function in four captive Indian elephants found relatively high platelet counts, with a mean of $637 \times 10^3/\mu\text{L}$.²⁰ Aggregation with ADP and collagen was less marked than in human platelet-rich plasma.²⁰ Asian elephant platelets were highly responsive to stimulation with platelet activating factor (PAF) and collagen, less responsive to ADP and non-responsive to arachidonic acid, serotonin, and epinephrine.⁹ The cyclooxygenase inhibitor, acetylsalicylic acid, produced no inhibition of either collagen or PAF induced aggregation.⁹ On ultrastructural examination, the unactivated elephant platelets contain large randomly distributed granules but lack the internal cristae that usually comprise the open canalicular system in many types of mammalian platelets.⁶ After PAF administration, large platelet aggregates form, but some platelets lack pseudopod formation or fusion of membranes while other behave as would be expected. The lack of platelet membrane fusion within the aggregates may permit the reversal of aggregation that is characteristic of the elephant platelet response to PAF.⁶

General coagulation studies have revealed that clotting factor concentrations are considerably higher in elephant whole blood than in human whole blood.^{14,20} The average factor VIII:C value in one study was 1.95 units/mL, approximately twice that of the human value.¹⁰ Human recombinant tissue factor was effective in activating the tissue factor-factor VII pathway, as

measured by the prothrombin time assay. Elephant factor XII was not readily activated by the commercial activated partial thromboplastin time (APTT) reagent formulated with a soluble activator. The average APTT results were twice as long as that of the human reference plasma.¹⁰ In contrast, and likely due to a difference in activating reagents, Indian elephant blood has been reported to clot rapidly, with APTT results less than in humans though similar to other mammals. Coagulation was reported to be less rapid in the African elephant.¹⁴ Relatively high fibrinogen concentrations, $4.6 \pm 0.5 \text{ g/L}$, have also been reported in elephants¹⁰ which are consistent with the findings presented in Table 120.2.

REFERENCE INTERVALS

Hematologic reference intervals are useful in health assessment and diagnosis of disease in exotic species, which frequently suppress their clinical signs. For many species, however, such reference intervals are lacking, as they are based on few samples, are produced from an inadequately described population of animals, and/or do not report laboratory methodologies. Though elephant blood has been studied for many years, the original reference intervals of elephant hematology were often generated from post-mortem samples with extensive transport times in the heat.^{5,8,36} Caution must be used when examining older reports or citations thereof and hemolysis must be considered when values seem abnormal.

In the twentieth century, reference intervals for elephants have varied significantly because of inaccurate differential cell counts where the different forms of monocytes were counted as lymphocytes. One study counted the bilobed and round cell type as lymphocytes in African elephant blood, which resulted in 60–80% relative lymphocyte counts.⁸ In another study, a review of Asian elephant blood revealed that nearly 30% of cell counts listed as lymphocytes were monocytes.²⁸ An additional study counted the bilobed cells as lymphocytes with relative counts of 73% lymphocytes and 3% monocytes.²⁰ Silva and Kuruwita counted the bilobed cells as monocytes, but counted all cells with round nuclei as lymphocytes;^{30,31} this resulted in

TABLE 120.2 Hematological Reference Intervals for Asian elephants (*Elephas maximus*)^a

	No.	Mean	S.D.	Reference Range
RBC ($\times 10^6/\mu\text{L}$)	20	2.9	0.4	2.1–3.6
Hgb (g/dL)	20	12.3	1.26	9.6–15.3
PCV (%)	20	35.7	3.79	27–44
MCV (fL)	20	125	7.42	111–138
MCHC (g/dL)	20	34.7	0.58	33.4–35.7
RDW	18	16.8	1.08	14.9–18.7
Fibrinogen (mg/dL)	20	400	137.17	100–600
Platelets ($\times 10^3/\mu\text{L}$)	18	513	98.43	342–719
WBC ($\times 10^3/\mu\text{L}$)	20	13.6	2.7	8.6–18.2
Bands/ μL	20	0	0	0.00–0.00
Segmented neutrophils/ μL	20	3180	820.59	1800–4300
Lymphocytes/ μL	20	2650	1456.64	990–5000
Monocytes/ μL	20	7140	2751.96	1600–12600
Eosinophils/ μL	20	220	172.94	0–580
Basophils/ μL	20	50	60.88	0–150
Plasma protein (g/dL)	20	8	0.66	6.7–9.3

^aComplete blood cell counts were performed using Cell Dyn 3500 (Abbott Diagnostics, Inc, Abbott Park, IL) with manual differentials using modified Wright-Giemsa staining. The reference intervals are based on $n = 25$ adult, predominantly female Asian elephants assessed as normal by physical exam and behavior over a 1 year time period. Animals were determined to be negative for tuberculosis.

relative lymphocyte counts of 38% and monocyte counts of 29%. Because monocytes with round nuclei are common in blood, this is likely still an overrepresentation of the lymphocyte population. Though these publications are older, inaccurate differential cell counts still regularly occur in practice today. When evaluating the published literature for reference intervals, one must pay particular attention to the standard deviation of the lymphocyte and monocyte counts. If the standard deviations are large, it is likely that inconsistent cell identification has been included in the reference interval. ISIS reference intervals, often cited as normal values for elephants, have lymphocyte and monocyte counts with large standard deviations and may be an example of inconsistent cell identification (see International Species Information System, <http://www.isis.org/CMSHOME/>).

The importance of accurate hematology reference intervals cannot be underestimated. Monocytosis can be an indicator of chronic inflammation in most species. Although monocytes generally have less potent bactericidal activity than neutrophils, they are important components of response to bacterial, viral, and fungal infections. Considering their predominance, it is likely that these cells play a crucial role in fighting infection in elephants. Highly variable, inaccurate reference intervals prevent the clinician from accurately assessing this important cell type. The authors have presented reference intervals (Table 120.2) for a small population of elephants that have been confirmed to be healthy with behavioral, physical, and laboratory examinations for over a 1 year time period. These reference intervals have cell counts that were performed based upon the cytochemical stains presented in this chapter.

The effects of age and gender differences on reference intervals have been examined. There have been

several studies that have looked for age and gender difference in hematologic reports.^{1,8} Except for the previously mentioned changes in pregnant females, none have been found. Studies of elephant calves (0–5 years) have consistently revealed increased WBC counts.^{14,23,36}

There have been anecdotal reports of clinical situations where accurate reference intervals may provide important information. For example, in case reports of endotheliotropic herpesvirus infection in an elephant, monocytosis was noted as part of this often fatal disease process.²⁷ Also monocytopenia has been reported in a case of *Streptococcus zooepidemicus* infection that led to pyelonephritis.²⁶

HEMATOLOGIC DISEASE

Tuberculosis

Tuberculosis is caused by bacteria within the *Mycobacterium tuberculosis* complex and can infect numerous mammalian species. Elephants are unique as one of the few species other than man that is primarily infected by *M. tuberculosis*, although infection with *M. bovis* has also occurred. *M. tuberculosis* is known to cause pulmonary and generalized disease in elephants and currently infects between 3% and 5% of elephants housed in captivity in North America.²¹

Hematology values that have been found to be significantly lower in tuberculosis infected animals include the mean corpuscular hemoglobin concentration. Hematologic values that were significantly higher in positive animals included band heterophils, eosinophils, and platelets.¹² Though clinicians frequently look for monocytosis as an indication of tuberculosis,

no significant difference in monocyte counts was found between culture positive and negative elephant samples.¹²

Elephant Endotheliotropic Herpesvirus

Both African and Asian elephants are the primary hosts for multiple species of elephant endotheliotropic herpesvirus (Betaherpesvirinae) and several newly described Gammaherpesvirinae.³⁵ Within the primary host, nonlethal vaginal, oral, cutaneous, and pulmonary lesions can be present. Of more clinical importance, individual elephants, most commonly juvenile Asian elephants, can present with a rapidly fatal hemorrhagic disease that is characterized by severe endothelial lesions. Deaths from elephant endotheliotropic herpesvirus are one of the leading causes of elephant calf mortality and have been documented in over 40 cases throughout the world.³⁵ Hematologic findings included mild leukopenia and anemia with more moderate to severe thrombocytopenia and lymphopenia in Asian elephants with fatal disease.²⁵ An elephant that was treated successfully in Missouri had a leukocytosis (up to $20 \times 10^3/\mu\text{L}$) with heterophilia and monocytosis but still had a lymphopenia.²⁷ Consensus polymerase chain reaction (PCR) may be used to screen whole blood as well as tissues at necropsy.

Blood Parasites

Trypanosomiasis, African sleeping sickness or surra, is caused by the protozoan *Trypanosoma* sp. in tropical regions. *T. evansi* has been reported in Asian elephants and *T. congolense* and *T. brucei* have been reported in African elephants.^{22,34} Hematologic abnormalities may be consistent with hemolytic anemia. Blood parasites are abundant, few, or absent in blood smears as in other species.

Filariasis, the presence of microfilaria in the blood, occurs in both species of elephants. In African elephants, thrombophlebitis and parenchymal liver lesions suggest that *Dipetalonema* sp. can cause hepatic disease.

SUMMARY

Due to the presence of a novel cell type (monocyte) in elephants, technologists require special education so that blood cells are identified and counted correctly. Definitive cell identification will allow for more accurate differential cell counts and thus more accurate reference intervals to be established, improving disease diagnosis and leading to better veterinary care of an endangered species.

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Hematology of Marine Mammals

THOMAS H. REIDARSON

Sample Collection
What is Normal?
Response to Infectious Disease

Response to Noninfectious Disease
Species Variation

Acronyms and Abbreviations

ACT, activated clotting time; AID, anemia of inflammatory disease; aPTT, activated partial thromboplastin time; CBC, complete blood count; EDTA, ethylenediaminetetraacetic acid; ESR, erythrocyte sedimentation rate; Hgb, hemoglobin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean cell volume; PCV, packed cell volume; WBC, white blood cell.

As we learn more about hematologic changes associated with various infectious and non-infectious diseases in marine mammals, we arm ourselves with tools to make crucial decisions about a patient's clinical condition. This would be impossible if not for years of routine clinical testing on marine mammals providing reference values for many of the marine mammals including beluga whales (*Delphinapterus leucas*), killer whales (*Orcinus orca*), captive and free-ranging bottlenose dolphins (*Tursiops truncatus*), hooded seals (*Cystophora cristata*), northern elephant seals (*Mirounga angustirostris*), Hawaiian monk seals (*Monachus schauinslandi*), Pacific walrus (*Odobenus rosmarus*), harp seals (*Phoca Groenlandica*), harbor seals (*Phoca vitulina*), Baikal seals (*Pusa sibirica*), California sea lions (*Zalophus californianus*), and Florida manatees (*Trichechus manatus latirostris*).^{1-5,7-9,11,16,23-25,28,30-32,35-36,38-40}

SAMPLE COLLECTION

For cetaceans, blood samples can be collected from the ventral fluke vasculature, ventral peduncle where the fluke veins converge, central vein of the dorsal fin, or interdigital flipper veins. Blood from pinnipeds can be drawn from the caudal gluteal vasculature or jugular in otariids (true sea lions), the extradural sinus for phocids (seals), and either extradural sinus or interdigital hind flipper veins in odobenids (walruses). Manatee blood

can be collected from the inter-radial-ulnar vasculature.³⁶ Blood can be collected from either the jugular or sublingual veins in polar bears. Blood can be collected from northern and southern sea otters from either the femoral or popliteal veins.³⁸

Blood is ideally drawn into collection tubes containing EDTA or heparin (for complete blood cell and white blood cell (WBC) differential counts), citrate (for blood clotting tests and fibrinogen), and thrombin clot activator tubes (for serum chemistry and iron tests). Because most cetaceans are factor XII (Hageman factor) deficient, the intrinsic pathway is not functional; therefore activated coagulation time (ACT) and activated partial thromboplastin time (aPTT) tests are inaccurate.²⁶ If serum is desired, blood from cetaceans should be collected into thrombin-containing blood tubes to stimulate clotting. When cetacean blood is collected into tubes without thrombin, clotting occurs very slowly and organizes poorly.

With blood volumes as small as 1.25–1.5 mL, complete hematologic and most serum chemistry tests can be analyzed using only whole blood from a citrate tube. After completing the complete blood count (CBC), the sample is centrifuged to harvest plasma for fibrinogen and serum chemistry tests. To compensate for dilution from the citrate solution, test values should be multiplied by a factor of 1.11 or 1.07 for 1.25 mL and 1.5 mL samples, respectively. Multipliers are not necessary for volumes greater than 1.5 mL (T.H. Reidarson,

unpublished observations). Unreliable values are obtained for calcium, sodium, and total carbon dioxide using this method, and erythrocyte sedimentation rate (ESR) tests cannot be run due to the small volume of blood. Although unconventional, the capacity to run this entire group of tests with small volumes of blood is valuable, especially when larger samples are difficult or impossible to obtain.

WHAT IS NORMAL?

When using published reference intervals or ranges, the clinician must make several assumptions about the health of the individuals in the dataset. Most authors define “clinically normal” individuals as appearing normal with respect to body mass, condition, and behavior, non-pregnant, and off medication for at least two weeks. Even with this rather strict definition some results fall outside statistical ranges, especially with small data sets. Using standard statistical methods most of these outliers are removed; however, certain ranges still appear broader than expected. This is especially true with wild populations and small datasets. It is therefore the responsibility of the clinician to determine the relevance of a value when it falls either in the high or low end of a range.

Having many years of cumulative data from individuals in a large collection, it is possible to evaluate where an individual fits in the overall species range and is conceivable for certain individuals to have statistically different values from the normal cohort. Individual differences can occur with white blood cell (WBC) counts, differential cell counts, mean corpuscular volume (MCV), ESR, iron, and fibrinogen. Explanations for these differences include age, sex, and health status. Therefore, it may be necessary to establish reference intervals for individuals from which to track health changes from normal status. When dealing with free-ranging animals or animals in a rehabilitation setting this approach is not practical, so reference means and intervals offer the best measure of the distribution of “normal” in each of these species. Tables 121.1A, B, 121.2A, B and 121.3 provide normal hematology values from a variety of marine mammal species.

RESPONSE TO INFECTIOUS DISEASE

The majority of diseases of marine mammals are from infectious agents and as such produce several inflammatory changes in the hemogram and leukogram. These indicators of inflammation include the WBC count and differential cell count, reticulocyte count, platelet count, hemoglobin (Hgb) (or packed cell volume [PCV]), fibrinogen, ESR, and iron.^{14,15,20}

In an effort to more closely detect the early stages of inflammation, we have altered the criteria for identification of band neutrophils. Rather than the more conventional definition (i.e. cells with pinched filaments greater than 50% of the filament diameter) we count any neu-

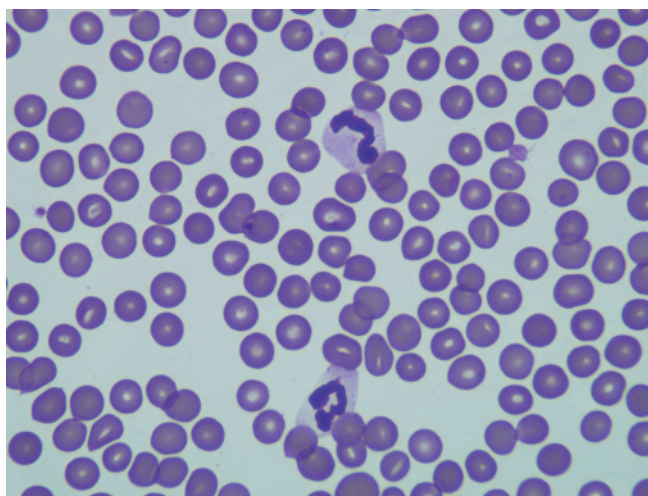


FIGURE 121.1 Blood smear from a killer whale (*Orcinus orca*) showing two “band” neutrophils. Any neutrophil with chromatin spanning the lobules is counted as a “band” even if the lobule is pinched greater than 50% of the diameter. Note that the neutrophil in the bottom would have been labeled a segmented neutrophil using the more traditional definition of a band. Wright’s stain; 100× objective.

trophil with chromatin spanning the lobules as a “band” (Fig. 121.1). The use of this definition permits the identification of marine mammals in early stages of band production before a left shift is present. Using this definition, 2–5% of WBCs are identified as “band” neutrophils in clinically normal marine mammals.

The vast majority of marine mammals with acute inflammation have decreased Hgb and PCV averaging 9–10%. These changes can be due to fluid shifts occurring in response to the release of inflammatory mediators. Once the inflammatory nidus is controlled, these parameters should return to normal, generally without evidence of reticulocytosis. If, on the other hand, Hgb and PCV slowly drop to subnormal levels, anemia of inflammatory disease (AID) may be the cause.

Anemia of inflammatory disease can be differentiated from blood loss anemia by a reticulocyte count. In AID, the reticulocyte count is low and in blood loss the reticulocyte count is increased even though the Hgb and PCV continue to drop. Reticulocyte counts vary between laboratories, as with other manually read tests (such as the differential cell count), so it is important to determine reference values for individuals under each veterinarian’s care.¹⁵

A recent study also demonstrated an increase in absolute platelet numbers in California sea lions as part of the leukocytic response, so as WBC and absolute neutrophil counts rise, an increase in platelet count should be expected. If platelet numbers are not increased during inflammation it should be considered clinically significant.¹⁴

TABLE 121.1A Normal Hematology and Protein Reference Intervals from Commerson's Dolphins, Common Dolphins, Beluga Whales, and Pilot Whales

	Commerson's Dolphin (<i>Cephalorhynchus commersoni</i>)	Common Dolphin (<i>Delphinus delphis</i>)	Beluga Whale (<i>Delphinapterus leucas</i>)	Pilot Whale (<i>Globicephala macrorhynchus</i>)
RBC ($10^6/\mu\text{L}$)	4.3–5.6	4.6–4.9	3.6–4.0	3.3–3.7
Hb (g/dL)	15.1–18.7	16.1–19.4	18.7–21.4	15.1–16
Hct (%)	44–52	46–55	51–59	43–45
MCV (fL)	93–105	100–114	160–183	123–129
MCH (pg)	33–37	35–40	59–67	43–46
MCHC (g/dL)	34–36	34–36	35–39	34–36
Platelets ($10^3/\mu\text{L}$)	109–261	55–100	64–148	70–90
Reticulocytes (%)	0.9–2.1	0.8–1.4	0.2–1.0	0.7–1.2
nRBC (%)	0–1.2	0	0–1	0
ESR (@ 60min)	0	0	0–9	16–52
Leukocytes ($/\mu\text{L}$)	3620–8160	4570–4900	5320–9560	4720–6500
Neutrophil ("band") ($/\mu\text{L}$)	0–163	0–98	0–382	0–5
Neutrophil (mature) ($/\mu\text{L}$)	1150–3250	2590–4150	2580–5520	2930–4360
Lymphocyte ($/\mu\text{L}$)	1260–2420	380–850	1100–4150	660–2080
Monocyte ($/\mu\text{L}$)	150–270	120–350	220–780	190–460
Eosinophil ($/\mu\text{L}$)	690–2200	620–1280	90–640	240–870
Basophil ($/\mu\text{L}$)	0	0	0	0
Plasma proteins (g/dL)	5.6–6.8	6.3–7.3	5.9–7.1	5.3–6.0
Albumin (g/dL)	3.5–3.9	3.9–4.7	4.0–4.8	2.9–3.3
Globulin (g/dL)	1.9–3.1	1.8–3.0	1.7–2.7	2.2–3.0
Fibrinogen (g/dL)	108–210	120–230	64–139	180–345

TABLE 121.1B Normal Hematology and Protein Reference Intervals from Pacific White-sided Dolphins, Killer Whales, False Killer Whales, and Bottlenose Dolphins

	Pacific White-sided Dolphin (<i>Lagenorhynchus obliquidens</i>)	Killer Whale (<i>Orcinus orca</i>)	False Killer Whale (<i>Pseudorca crassidens</i>)	Bottlenose Dolphin (<i>Tursiops truncatus</i>)	
				Free-ranging	Captive
RBC ($10^6/\mu\text{L}$)	5.2–6.0	3.4–4.2	3.1–4.7	3–4.1	3.0–4.0
Hb (g/dL)	17.0–20.6	13.5–15.9	13.3–18.1	12.4–15.4	12.6–15.8
Hct (%)	48–56	39–46	39–51	37–47	36–46
MCV (fL)	90–98	103–117	104–120	106–134	108–136
MCH (pg)	33–35	36–42	36–44	35–44	37–47
MCHC (g/dL)	34–36	34–36	33–37	30–35	32–38
Platelets ($10^3/\mu\text{L}$)	78–162	98–228	58–154	92–238	58–178
Reticulocytes (%)	0.8–2.3	0.5–2.5	0–3.7	N.T. ^a	0.3–3.5
nRBC (%)	0–0.7	0	0–1	0	0–3
ESR (@ 60min)	0	0–2	3–29	N.T.	0–30
Leukocytes ($/\mu\text{L}$)	2530–6930	3760–7890	4450–9350	5900–13700	4480–9080
Neutrophil ("band") ($/\mu\text{L}$)	0–139	0–237	0–374	0	98–522
Neutrophil (mature) ($/\mu\text{L}$)	1250–3730	2380–6060	2280–5040	2450–6850	2460–5980
Lymphocyte ($/\mu\text{L}$)	390–1390	520–1850	990–2490	310–3810	420–2500
Monocyte ($/\mu\text{L}$)	80–240	140–420	120–400	80–640	110–430
Eosinophil ($/\mu\text{L}$)	720–1910	8–160	410–1540	1070–5340	130–1510
Basophil ($/\mu\text{L}$)	0	0	0	0–50	0
Plasma proteins (g/dL)	5.7–6.9	5.5–6.6	5.8–6.6	6.3–8.7	5.6–7.6
Albumin (g/dL)	3.0–3.8	3.0–3.7	3.4–3.8	2.8–3.7	4.2–5.4
Globulin (g/dL)	2.4–3.4	2.0–3.4	1.7–3.3	3.0–5.5	1.0–2.6
Fibrinogen (g/dL)	147–253	122–246	176–340	N.T.	140–260

^aN.T., not tested.

TABLE 121.2A Normal Hematology and Protein Reference Intervals from Captive Northern Fur Seals, Steller Sea Lions, Northern Elephant Seals, and Harp Seals

	Adult Northern Fur Seal (<i>Callorhinus ursinus</i>)	Adult Steller Sea Lion (<i>Eumetopias jubata</i>)	Juvenile Northern Elephant Seal (<i>Mirounga angustirostris</i>)	Adult Harp Seal (<i>Pagophilus groenlandicus</i>)
RBC ($10^6/\mu\text{L}$)	4.4–5.8	4.1–5.2	2.0–2.9	4.2–5.3
Hb (g/dL)	15.1–18.9	15–19	17.3–24.9	18.5–24.8
Hct (%)	42–53	42–49	38–55	45–63
MCV (fL)	92–102	96–111	170–199	97–125
MCH (pg)	31.7–36	33–39	70–102	39–49
MCHC (g/dL)	32–38	32–36	40–53	36–44
Platelets ($10^3/\mu\text{L}$)	320–698	196–489	120–754	159–755
Reticulocytes (%)	0	N.T.	N.T.	N.T.
nRBC (%)	0	0	0	0–4
ESR (@ 60 min)	N.T. ^a	N.T.	N.T.	N.T.
Leukocytes ($/\mu\text{L}$)	3400–11500	4700–10200	9840–28600	8600–15030
Neutrophil (“band”) ($/\mu\text{L}$)	0–264	0–264	431–517	0–328
Neutrophil (mature) ($/\mu\text{L}$)	2173–7738	2420–8568	4970–21890	4386–9512
Lymphocyte ($/\mu\text{L}$)	534–3960	584–2886	510–6170	1440–3608
Monocyte ($/\mu\text{L}$)	92–1221	134–876	20–2750	310–1376
Eosinophil ($/\mu\text{L}$)	0–1700	0–378	0–960	453–2460
Basophil ($/\mu\text{L}$)	0–240	0	0	0
Plasma proteins (g/dL)	6.3–7.6	7.8–8.8	5.4–8.6	6.3–7.4
Albumin (g/dL)	2.5–3.5	3.7–4.2	2.6–4.2	3.2–3.5
Globulin (g/dL)	3.7–4.5	3.7–5.0	2.0–5.1	3.0–4.7

^aN.T., not tested**TABLE 121.2B** Normal Hematology and Protein Reference Intervals from Adult Harbor Seals, Gray Seals, and California Sea Lions

	Harbor Seal (<i>Phoca vitulina</i>)	Gray Seal (<i>Halichoerus grypus</i>)	California Sea Lion (<i>Zalophus californianus</i>)
RBC ($\times 10^6/\mu\text{L}$)	4.7–5.1	3.5–4.9	4.2–4.9
Hb (g/dL)	19.4–20.8	16.4–20	15.7–19.3
Hct (%)	54–59	43–52	47–56
MCV (fL)	111–119	104–129	108–112
MCH (pg)	39–43	40–47	36–43
MCHC (g/dL)	35–37	35–43	34–37
Platelets ($\times 10^3/\mu\text{L}$)	285–355	244–519	218–390
Reticulocytes (%)	0.3–0.5	N.T. ^a	0.2–0.5
nRBC (%)	0	0	0
ESR (@ 60 min)	3.0–11	N.T.	8.0–38
Leukocytes ($/\mu\text{L}$)	7100–8900	6800–13600	4700–6100
Neutrophil (“band”) ($/\mu\text{L}$)	0–157	0–360	0–225
Neutrophil (mature) ($/\mu\text{L}$)	4690–6490	4278–9559	2690–3800
Lymphocyte ($/\mu\text{L}$)	1620–2450	1666–3872	1060–2040
Monocyte ($/\mu\text{L}$)	520–910	192–1599	200–440
Eosinophil ($/\mu\text{L}$)	100–490	0–994	100–490
Basophil ($/\mu\text{L}$)	0	0–396	0
Plasma proteins (g/dL)	7.7–8.2	7.8–9	6.8–7.4
Albumin (g/dL)	2.6–3.0	3.3–4.2	3.3–3.6
Globulin (g/dL)	4.4–5.3	3.9–5.4	3.6–4.1
Fibrinogen (g/dL)	146–234	N.T.	156–316

^aN.T., not tested.

TABLE 121.3 Normal Hematology and Protein Reference Intervals of Florida Manatees, Polar Bears, and Sea Otters

	Florida Manatee (<i>Trichechus manatus latirostris</i>)	Polar Bear (<i>Ursus maritimus</i>)	California Sea Otter (<i>Enhydra lutris</i>)		
			Pups	Captive adults	Wild adults
RBC ($10^6/\mu\text{L}$)	2.4–3.4	5.4–8.2	2.5–4.6	4.6–6.0	4.3–5.7
Hb (g/dL)	9.8–13.2	12.9–17.5	6.1–17.7	15.6–22.2	15.1–21.2
Hct (%)	30–40	36.1–53.3	23–51	46–62	48–64
MCV (fL)	122–149	62.4–72.5	82–114	92–120	95–123
MCH (pg)	38–46	21.2–25.6	24–42	30–42	30–42
MCHC (g/dL)	30–33	328–370	28–38	29–43	30–42
Platelets ($10^3/\mu\text{L}$)	195–412	60–1083	N.T. ^a	N.T.	N.T.
Reticulocytes (%)	0–4	0	N.T.	N.T.	N.T.
nRBC (%)	0	0	N.T.	N.T.	0–3.0
ESR (@ 60min)	7.0–85	N.T.	N.T.	N.T.	N.T.
Leukocytes ($/\mu\text{L}$)	4000–11800	3300–10800	1100–14440	4300–15160	3670–14530
Neutrophil (“band”) ($/\mu\text{L}$)	0	100–300	0	0	0
Neutrophil (mature) ($/\mu\text{L}$)	960–5990	1800–4900	2150–10160	1530–10350	1240–10060
Lymphocyte ($/\mu\text{L}$)	960–8590	600–1700	1000–4330	1600–5320	1050–4780
Monocyte ($/\mu\text{L}$)	0–1020	300–1120	0–1880	0–1080	0–1100
Eosinophil ($/\mu\text{L}$)	0	40–440	0–930	0–1020	0–1160
Basophil ($/\mu\text{L}$)	0	0–130	0	0	0
Plasma proteins (g/dL)	6.2–8.6	6.5–9.1	3.3–7.5	6.0–8.2	5.9–8.1
Albumin (g/dL)	3.6–5.9	3.7–5.2	1.3–3.5	2.8–4.0	2.4–3.5
Globulin (g/dL)	2.6–2.7	2.3–4.2	1.0–5.0	2.7–4.7	3.1–5.0

^aN.T., not tested.

During the acute phase of a bacterial infection, serum iron can drop rapidly and dramatically. Iron is sequestered by iron-binding proteins and temporarily stored in the liver, making it less available to the pathogenic bacteria. Acute phase proteins (e.g. interleukin-1, interleukin-6, C-reactive protein, prostaglandins, tumor necrosis factor, and interferon) help mediate this iron sequestration.⁵ The opposite is true of hepatocellular bacterial infections and even some noninfectious diseases, such as hemochromatosis, lipidosis, and azole hepatopathies, where iron is liberated from the liver and into the serum.¹⁹ Trends in serum iron levels are important when evaluating clinical condition and prognosis; however, the change in magnitudes do not appear to correlate directly with severity.

In our experience, a quantitative test for plasma fibrinogen is the most reliable indicator of inflammation. Qualitative heat precipitation methods are not as accurate. Some clinicians believe fibrinogen correlates directly with ESR; however, in our practice, we have seen variable effects. Although we frequently observe an increased ESR when the fibrinogen is increased, the converse is frequently not true. In dehydrated patients, increased viscosity slows the sedimentation of RBCs, but has little or no effect on the concentration of fibrinogen.

RESPONSE TO NONINFECTIOUS DISEASE

There are a number of noninfectious conditions that produce the hallmark features of inflammation, thus making them nearly indistinguishable from infectious diseases. These include certain types of neoplasia, trauma, and reproductive abnormalities. Neoplasia such as urogenital carcinoma of California sea lions and extensive sublingual squamous cell carcinoma of bottlenose dolphins is capable of inciting a systemic inflammatory response and, even though a gamma herpes virus has been linked to the urogenital cancer, its contribution to inflammation is minor compared to the necrosis associated with tumor growth and invasion of internal organs (J. St. Leger, personal communication).

Trauma, especially involving muscles or gastrointestinal tract, and reproductive abnormalities, such as fetal distress, premature birth, or abortion, lead to an inflammatory reaction from tissue damage. Release of tissue thromboplastins, RBC fragments, peroxidases, and other muscle cell membrane fragments can initiate an inflammatory response.³⁴ Ensuing shock can compromise the gastrointestinal mucosa leading to translocation of bacteria and toxins across the mucosa.^{11,13,33} Any of these processes can also stimulate production of local and systemic cytokines and other inflammatory mediators.

Inflammatory changes are frequently seen in CBCs of females in the last 2–3 weeks of gestation.^{27,29} In our experience, these include all of the parameters mentioned except reticulocyte count. If these changes are seen earlier in pregnancy, they signify fetal distress, premature birth, or abortion. In all cases it is difficult to determine whether inflammation is the result of an infectious agent or the cascade of events involved in pregnancy termination. Implantation, endometrial development, abortion, and parturition are mediated by hormones, interleukins, and cytokines. The main initiators of inflammation appear to be interleukin-6 and C-reactive protein that stimulate cervical ripening and softening.

SPECIES VARIATIONS

In cetaceans, beluga whale MCV values are significantly greater than those of other species, and pilot whales and false killer whales have elevated ESR compared to cetacean species. To our knowledge, the only cetacean species with a zero ESR at 60 minutes are killer whales, common dolphins (*Delphinus delphis*), Pacific white-sided dolphins (*Lagenorhynchus obliquidens*), and Commerson's dolphins (*Cephalorhynchus commersoni*). Statistically significant differences between free-ranging and captive bottlenose dolphins include greater MCV, platelet count, lymphocyte and eosinophil numbers, ESR, and globulins in the former.^{5,21} The only statistically significant sex difference was lymphocyte numbers in captive bottlenose dolphins where females had greater numbers.

Of the 10 species of pinnipeds examined, the only remarkable differences include increased MCV, mean

corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and lower RBC numbers for northern elephant seals (*Mirounga angustirostris*) (Fig. 121.2).²¹ Known for being the deepest divers (over 1,500 meters) these differences are presumed to be adaptations for breath holding.²² Fewer, larger RBCs with higher MCHC, and hence elevated oxygen storage, accompanied by reduced blood viscosity and reduced flow resistance near stasis suggest that this feature of phocid seal blood is an adaptation to circulatory redistribution during long dives. Furthermore, these changes occur early in life as juveniles reach weanling age, with the development of cardiorespiratory changes related to diving.^{6,10,12,37}

Marked pressure changes are known to activate human platelets.¹⁷ However, killer whale platelets are not altered during or after diving to great depths, nor do they experience abnormal bleeding associated with platelet dysfunction.¹⁸ Thromboxane, a known platelet aggregating agent in mammals, is produced in normal amounts in killer whales; however, killer whale platelets are less responsive to thromboxane.⁴⁰ This phenomenon may serve a protective role in these mammals, and perhaps other deep divers such as northern elephant seals, by preventing thrombosis during diving and resurfacing.

The predominant segmented nucleated cell type of the Florida manatee contains small, pleomorphic, rod to round, eosinophilic cytoplasmic granules. As such, this cell is termed a heterophil or heterophilic granulocyte. However, recent evidence has shown this cell to be biochemically related to the neutrophil (Fig. 121.3).³⁶

Other unique features of marine mammal hematology include the presence of codocytes in 25–50%

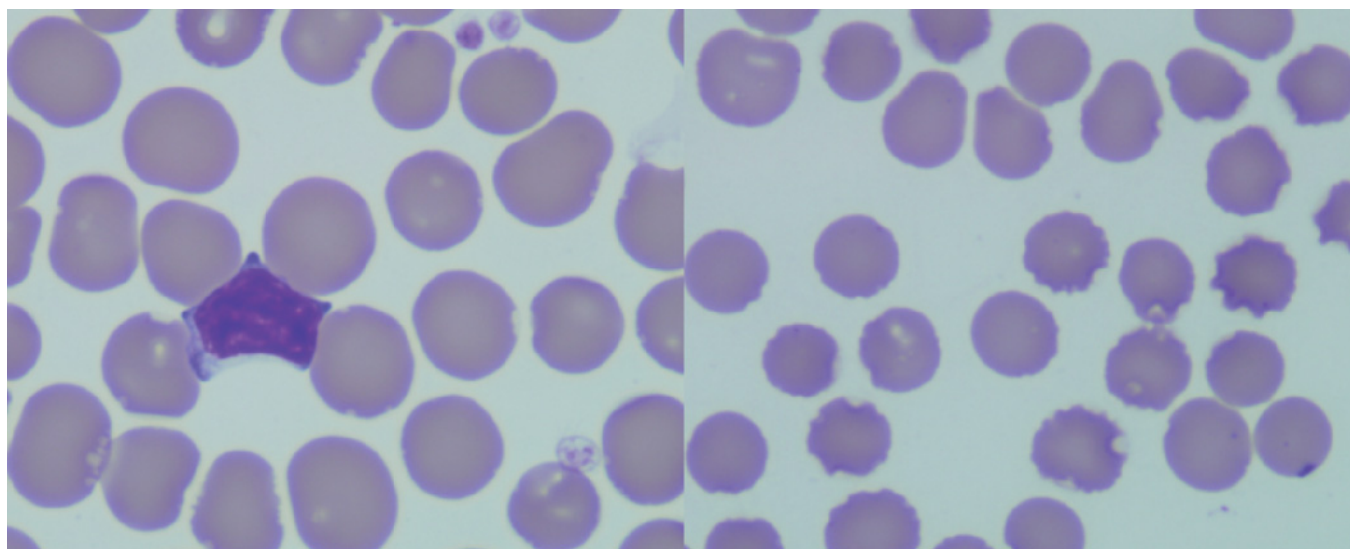


FIGURE 121.2 Blood smears comparing RBCs of (A) a northern elephant seal (*Mirounga angustirostris*) with (B) a California sea lion (*Zalophus californianus*). Northern elephant seals have the largest MCV of any mammal at greater than twice the size of the California sea lion. Wright's stain.

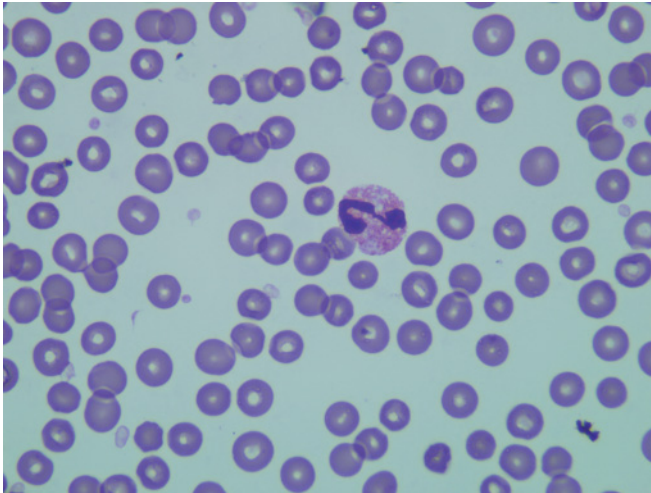


FIGURE 121.3 Heterophil from a Florida manatee (*Trichechus manatus latirostris*). Wright's stain; 100× objective.

of normal walrus RBCs, hypersegmented neutrophils in greater than 50% of normal harbor seal blood smears, and Howell-Jolly bodies in 1–5% of all species of normal cetacean RBCs (T.H. Reidarson, unpublished data).

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Hematology of Chickens and Turkeys

PATRICIA S. WAKENELL

Blood Collection
 Cell Counts and Staining
 Hematopoiesis
 Hematopoietic Growth Factors
 Erythrocytes
 Granulocytes
 Heterophils

Eosinophils
 Basophils
 Monocytes
 Lymphocytes
 Thrombocytes
 Normal Reference Intervals for Chickens and Turkeys

Acronyms and Abbreviations

ATP, adenosine triphosphate; CO₂, carbon dioxide; EDTA, ethylenediaminetetraacetic acid; FMLP, formyl-methionyl-leucylphenylalanine; H:L, heterophil:lymphocyte ratio; IL, interleukin; iNOS, inducible nitric oxide; IP5, inositol pentaphosphate; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MHC, major histocompatibility complex; N:C, nuclear:cytoplasmic ratio; NMB, new methylene blue; NO, nitric oxide; PAS, periodic acid-Schiff; PCV, packed cell volume.

Although the chicken has been used as the research animal model for establishing normal parameters for other avian species, little information has been published on hematology of chickens and turkeys in a clinical setting. The chicken and turkey have recently become more popular as backyard “pets” and visits to the veterinary hospital are increasing. Most of the current information about routine hematologic parameters is extricated from clinical values established for psittacines. Although serology is the predominant method of disease monitoring in commercial poultry, examination of blood smears, bone marrow and clinical chemistry values is rarely done. The genetics, body size and production purpose of chickens and turkeys is highly divergent and influence disease resistance as well as production parameters.⁵⁷ Commercial broiler chickens have increased normal Pco₂ when compared to laying chickens (P.S. Wakenell, personal observation) and macrophage inducible nitric oxide (iNOS) is influenced by genetic lineage.^{38,44,45,55} The following information has been obtained primarily from the White Leghorn research chicken, and the influence of genetics, reproduction, geographic location, breed and season are just now being explored.³⁷

BLOOD COLLECTION

The most common location for venipuncture in both the chicken and the turkey is the wing (brachial) vein. Size of the bird and bird type (show, game or fighting chicken) may dictate use of the jugular vein or the medial metatarsal vein. Cardiac puncture can be used by highly skilled personnel or in cases where the bird is euthanized immediately after blood collection.

The wing vein is superficial and easily visualized in adult birds and chicks and poults older than 1.5–2 weeks of age. Either wing can be used and collection can be performed with or without assistance. To prevent flapping and kicking, control of the unused wing and feet is essential. This is usually achieved by holding the bird in one arm with the bird’s feet facing behind the upper arm toward the rear, pinning the unused wing between the bird’s body and the collector’s trunk and outstretching the target wing with the holding arm. The bird’s head generally hangs down which has a calming effect. This method leaves the collector one hand free to collect the blood but also can be used when assisting others. Most chickens and turkeys need a short time to relax after holding but then will remain calm throughout the procedure. For heavy birds, the venipuncturist

may need to kneel during the procedure with the bulk of the bird's weight resting on the handler's leg, or have assistance. The underside of the wing is generally free of most feathers over the vein and the remainder can be plucked without distress. The vein can be accessed by either directing the bevel of the needle toward the bird's body or toward the wing tip. The vein does not need to be held off either before or after the procedure as high levels of tissue thromboplastin induce rapid clotting, which can be problematic for producing good samples. However, if blood on the wings and body are upsetting to the owner or may result in cannibalism, pressure should be applied to the site after collection. It is best to enter the skin first and then access the vein. The vein is superficial so needle placement is best at halfway between joints and almost horizontal with respect to the wing. Hematomas result even with clean procedures.

For highly aggressive breeds (fighting chickens), heavy birds (tom turkeys) and newly hatched birds, the right jugular vein is used. Highly aggressive breeds often cannibalize their wing if there is blood spotting and/or hematomas. The right jugular vein is more superficial than the left jugular vein and the hematoma formation is less than with wing vein puncture. Small birds can be held in one hand with the head held between the fingers and the neck outstretched. No feather plucking is necessary as the adjacent feather tracts can be parted to visualize the vein.

Cardiac puncture is used in situations where the bird will be euthanized immediately after the procedure and is commonly used for assessing maternal antibody levels in newly hatched chicks. Due to the risk of damaging the lungs and expectoration of blood, this technique must only be used by trained personnel if birds are not euthanized. The bird is held upside down by the feet in one hand with the bird's back supported by the holder's arm. With heavy weight birds (broilers and turkeys), the bird is laid on a table in a dorsal recumbent position with the feet held by one hand. The thoracic inlet is palpated and the needle is placed through the inlet without angling to either side and horizontal to the bird's backbone. The length of the needle placement and needle depends on the size of the bird.

An alternate location to those described above is the medial metatarsal vein which is located on the medial side of the tarsometatarsal bone. This site is used in half grown and older chickens and turkeys weighing approximately 300 grams or more. Since hematomas usually do not occur in this area, this site is ideal for situations where multiple blood samples need to be collected over a short time period.

As with psittacines (Chapter 123), the maximum volume of blood collected is 1% of the bird's body weight. This is generally not a limiting factor for turkeys and chickens as with small psittacines and passerines. Birds are more tolerant to blood loss than mammals, probably due to volume replacement from tissue fluids and, in some species, baroreceptor reflexes.³ Vacuum tubes exert too much pressure on the vein causing collapse and are not recommended.

CELL COUNTS AND STAINING

Although anticoagulants, particularly heparin, can cause artifacts in blood smears, it is generally impractical to do a blood smear immediately after blood collection.⁵ Blood can be collected in a syringe coated with anticoagulant, traditionally ethylenediaminetetraacetic acid (EDTA) for avian samples, or in an uncoated syringe with blood transferred to a coated collection tube. Although EDTA has historically been preferred for avian samples, a recent study showed that the differences between differentials done on heparinized samples versus EDTA samples were minimal.¹⁸ Transferring to a coated tube allows more control of the ratio of the blood volume to anticoagulant, particularly in cases where small quantities are drawn. The standard two slide pushing technique can often result in a high number of smudge cells as some avian cells tend to be more susceptible to rupture than mammalian cells.⁴¹ When using this technique, sufficient numbers of smears need to be produced to accommodate loss of imperfect smears. Two alternate methods can be used: (1) Place a drop of blood onto a slide and place a coverslip over the drop. Horizontally remove the coverslip as the blood drop spreads.⁴¹ This method sometimes results in overly thick viewing areas and granule shape can be difficult to ascertain. (2) Put a small drop of blood on a coverslip and use a Cytospin centrifuge to distribute the drop evenly around the coverslip. Smears using all three methods need to be scanned to determine the percentage of smudge cells in relationship to 100 counted cells. The traditional stains used for mammalian smears can also be used for avian blood smears. Recently a new staining method specifically designed for avian cells has been described.²⁴ Using this staining method, white blood cell color differences help cellular identification in addition to size and shape.

Leukocyte counts using the eosinophil Unopette 5877 (Becton-Dickinson, Rutherford, NJ) or Natt-Herrick's solution are considered the gold standard for counting avian samples.⁴¹ Automated systems, while useful in some situations such as determining the heterophil/lymphocyte ratio (H:L),⁴³ are not accurate for differentiating all cell types.³⁰ Flow cytometry has been successfully used and has been shown to accurately separate lymphocytes from thrombocytes, but may not be practical in a clinical setting.^{2,33} Manually counting cells from a blood smear is often inaccurate and is only recommended for situations where other methods are not available. Since some cell types can be preferentially lysed when using the two slide pushing technique, it is important to use only good quality smears. Counting the number of leukocytes observed in 2–4 high power fields each on at least five smears helps increase accuracy. If limited to one smear, count the number of leukocytes per high power field on at least 10 fields, average, and multiply by 2000.

Erythrocytes can be counted using an erythrocyte Unopette (Becton-Dickinson) method or by using an automated cell counter. Both leukocytes and erythrocytes will be counted using the automated counters but

the leukocyte numbers are small in comparison with the erythrocytes and do not skew erythrocyte counts. Packed cell volume (PCV) is determined by using the microhematocrit method. Hemoglobin can be determined using the same method used for mammalian species but the sample needs to be centrifuged before obtaining the optical density to remove nuclei after cell lysis. Mean corpuscular hemoglobin (MCH), MCH concentration (MCHC), and mean corpuscular volume (MCV) can be calculated. Reticulocytes can be estimated after staining with new methylene blue (NMB). Residual cytoplasmic RNA stains basophilic with NMB. A reticulocyte count of approximately 3% can be seen in normal healthy chickens.

Often attempts are made to remove thrombocytes from blood samples, using flow cytometry in order to reduce interference with lymphocyte counting.² Using the same method, thrombocytes can be preferentially sorted after labeling with monoclonal antibodies.^{1,54} Thrombocyte numbers can also be estimated on blood smears and each oil immersion field should have 1–3 thrombocytes. Since thrombocytes tend to clump and be dragged farther out on the feathered edge, visual estimation is difficult and numbers are usually reported as decreased, increased or normal.

HEMATOPOIESIS

Most research on ontogeny of the hematologic and lymphoid systems has been done in the chicken and quail, frequently by using chicken quail chimeras.^{34,42} Lymphoid, erythroid and probably thrombocyte precursor cells start in intraembryonic mesenchyme in the para-aortic region around 2–3 days of embryonation.^{4,6,46} Primitive lymphoid stem cells travel to the yolk sac between days 2 and 7 of embryonation and are first found in the yolk sac on day 7. The earliest the prebursal and prethymic stem cells are detected is day 5, but it is speculated that they are present starting day 2.^{11,46} This is the same site identified as the origin of mammalian B cells.

The thymus is populated in three waves (chicken and quail) by extrinsic stem cells from the yolk sac (probably also the spleen, and by embryonation day 4, the thoracic aorta as well may directly contribute).¹¹ The avian liver is not an important transient site for prethymic stem cells as in mammals. The liver is mainly for extramedullary hematopoiesis and prebursal stem cell “transients.”

The bursa is first observed as an outgrowth of the urodeal membrane (base of the posterior cloaca) at 3–4 days of embryonation.⁴⁶ Epithelial cells in the bursa attract bursal stem cells to the bursa. Entrance to the bursa is regulated at the level of the endothelial cells of the bursa microvasculature. Epithelial cells retain the ability to attract lymphocytes even after involution. Bursal stem cells from the yolk sac or other sites of hematopoiesis such as the spleen, liver, bone marrow, and blood seed the bursa starting around 7–10 through 15 days of embryonation. Those that remain outside of

the bursa (most of the population) undergo apoptosis. B lymphocytes from the bursa seed secondary lymphoid organs just prior to and post hatch. The bursa is required for generation of antibody diversity and functional maturation of the B cell and the thymus is required for T cell maturation. Major histocompatibility complex (MHC) “dosage” appears to modulate the cellularity of both the bursa and the thymus.⁹ Chickens have been produced that have three and four copies of the MHC microchromosome. These chickens have decreased bursal, thymus and body weight. Both the bursa and the thymus involute at sexual maturity and it is unknown where the post-bursal and thymic “stem” cells that presumably maintain the B and T cell pool after involution are located. There are three populations of B lymphocytes in the periphery. A cell surface antigen, LT2, which is on all precursor cells, is used to help sort these populations.

1. Short lifespan (3 days): the peripheral blood lymphocyte concentration is 60%, comprise 90–95% of bursal emigrants, do not divide in periphery, come from the cortex, have high levels of LT2 which decrease with age, disappear after involution.
2. Longer-lived (2 weeks): 35% of peripheral blood lymphocytes, do not divide in periphery, 5–10% of bursal emigrants.
3. Indefinite lifespan (unable to document through surgical bursectomy): divide rapidly, lifespan of individual cells is probably short but cells are always being replaced, population in blood increases with time, “post bursal stem cells.”⁴⁶

The role of the bone marrow in avian lymphopoiesis is unclear. It is probably not a primary organ as in mammals but may contain a small population of post-bursal and thymus “stem” cells that provide a source of self renewal. B cells migrate into thymus after hatching and the thymus behaves as a secondary organ which is not found in mammals.

Spleen germinal centers are B cell dependent but T cells are required for formation. Spleen germinal center formation is age dependent and reaches the maximum number at 4–5 weeks of age. Intestinal germinal centers are not age dependent and continually increase.

Erythroid and thrombocyte stem cells (no megakaryocytes) also colonize the yolk sac and hematopoietic activity peaks around 10–15 days of embryonation. Hematopoietic activity persists in the yolk stalk of hatched chicks. Erythrocytes and thrombocytes are formed in the vascular sinuses and granulocytes are formed extravascularly.³ Erythrocytes and thrombocytes and their precursors are predominant in the circulating blood of the embryo, with leukocyte precursors being less common. After hatch, hematopoiesis occurs in many visceral and nonvisceral organs but is primarily located in the marrow of the vertebrae and long bones. Limited hematopoietic activity can be observed in the heart and serosa of the small intestine shortly after hatch. More extramedullary hematopoiesis is observed in avian species other than the chicken, and the liver is the most common location. Granulopoiesis

is the most predominant hematopoietic activity in the spleen before hatch, with lymphopoiesis predominating after hatch.³ Bone marrow occurs in conjunction with medullary bone. The extravascular space in the marrow also contains fat and lymphoid aggregates. The avian bone marrow is predominantly dedicated to producing erythroid cells: the number of reserve mature granulocytes is very low compared to mammals.

The ultrastructural anatomy of the embryonic and adult bone marrow in chickens is different from that of mammals.^{3,5} Capillary branches from marrow arterioles empty into medullary sinuses. Unlike capillaries, these sinuses are lined by endothelium without a basement membrane. The endothelium is continuous with numerous intercellular junctions between immature erythrocytes and endothelial cells. This adherence to the endothelium is thought to play a role in preventing immature erythrocytes from entering the circulation and in regulating erythropoiesis. Thrombocyte precursors have not been definitively identified in avian bone marrow, presumably due to their fragility and subsequent loss during processing, but they are also believed to occur in the intravascular sinuses. Early thrombocyte precursors may be indistinguishable from erythrocyte precursors. Stem cells observed migrating through endothelium between intravascular and extravascular sinuses likely represent migration of the pluripotent stem cell to the extravascular sinus. Maturing granulocytes also migrate through the endothelium and may undergo final maturation extravascularly. Monocytogenesis is not apparent in avian marrow, and early monocyte precursors are likely indistinguishable from early granulocyte precursors. Macrophages are not prominent in avian bone marrow.

The spleen also plays an important role in hematopoiesis, particularly with lymphopoiesis and granulopoiesis, as mentioned previously. The spleen is located at the junction of the proventriculus-gizzard and the shape is variable within avian species. It is oval in the chicken and turkey and brick red in color.²³ Accessory spleens can sometimes occur in various locations in the abdominal and, to a lesser extent, thoracic cavities and are considered normal. The red and white pulp have less distinct separation than in mammals and there is indistinct connective tissue trabeculae. The circulation is open with no direct connections between arteries and veins. The spleen has sheathed capillaries (Schweigger-Seidal sheaths) which appear as pale areas on microscopic examination.^{17,36} These sheaths differ in prominence depending on species, with owls having the largest. Capillaries are surrounded by macrophages with phagocytic capabilities. White pulp is diffuse with occasional germinal centers and is concentrated primarily around arteries and sheaths.

HEMATOPOIETIC GROWTH FACTORS

The identification and description of avian cytokines and lymphokines has expanded exponentially in recent years.^{15,25} Due to the general lack of homology between

avian and mammalian cytokines, use of mammalian monoclonal antibodies for identification of avian cytokines resulted in limited information. Most of the research has been done with chickens, ducks and turkeys. To date, the following cytokines have been either fully or partially characterized in avian species: erythropoietin,¹⁹ interleukin-1 (IL-1),³⁵ IL-2,²⁹ IL-6,⁴⁹ IL-8,⁵⁶ IL-10,⁴⁷ IL-12,⁸ IL-15,²⁹ IL-16,³² IL-17,²⁰ IL-18,¹⁶ granulocyte colony stimulating factor,⁴⁸ thrombocyte inhibitory factor,³ and tumor necrosis factor.³⁹

ERYTHROCYTES

Avian erythrocytes are oval, nucleated cells with round immature form. Maturation of erythrocytes is outlined in Table 122.1. Mature chicken and turkey erythrocytes found in the peripheral blood are large elliptical cells measuring approximately $12 \times 6 \mu\text{m}$. They have homogeneous eosinophilic cytoplasm and a central round to oval nucleus with a condensed chromatin pattern. Because erythropoiesis is intravascular or intrasinusoidal, occasional rubricytes can be found in the peripheral blood of healthy birds (Fig. 122.1). The percentage of reticulocytes seen in the peripheral blood of normal chickens and turkeys is somewhat higher than in most mammals. Polychromasia is more prominent in younger birds, but typically does not exceed 5%. Common artifactual abnormalities in erythrocyte morphology include cytoplasmic refractile vacuoles, smudge cells, and various morphologies induced by stretching cells while preparing the smear such as spindling, bilobed nuclei, bare nuclei and erythroplastids (anucleate fragments of erythrocyte cytoplasm).

TABLE 122.1 The Erythrocyte Maturation Sequence³

Cell Maturation Stage	Description
Rubriblast	Large cell, central round nuclei, granular chromatin, prominent nucleoli, very basophilic cytoplasm with mitochondrial spaces
Prorubricyte	Nucleoli and mitochondrial spaces inapparent
Basophilic rubricyte	Clumped chromatin pattern, very basophilic cytoplasm
Early polychromatic rubricyte	Cytoplasm becoming grayish, indicating hemoglobin production, chromatin more clumped, smaller nucleus
Late polychromatic rubricyte	More grayish to eosinophilic cytoplasm, clumped chromatin, small round nucleus
Polychromatic erythrocyte	Similar to mature erythrocytes but slightly larger with a more basophilic cytoplasm; should be 1–5% of circulating erythrocytes
Mature erythrocyte	Homogeneous eosinophilic cytoplasm, oval cell with central oval nucleus and condensed chromatin pattern

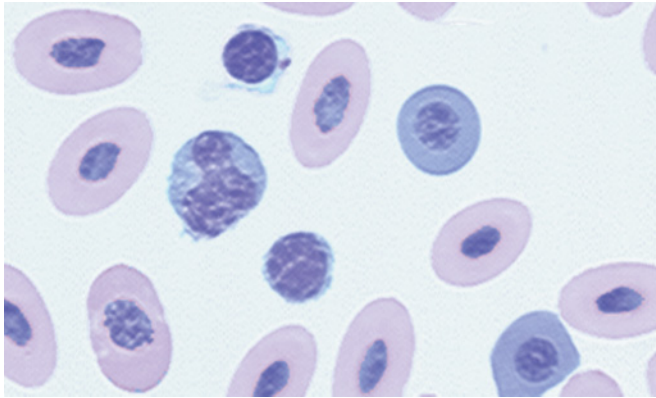


FIGURE 122.1 Thrombocyte with cytoplasmic azurophilic granule at one pole, two rubricytes, small lymphocyte, monocyte. Cells are identified in a clockwise manner. Wright-Giemsa stain; magnification 100 \times .

Avian erythrocytes function similarly to mammalian erythrocytes with some biochemical differences.¹⁰ Like fish erythrocytes, avian erythrocytes can participate in the immune response by producing cytokine-like factors.⁴⁰ The structure of avian hemoglobin, however, is similar to mammals. 2,3-Bisphosphoglycerate is present only in chicken embryonal erythrocytes and disappears shortly after hatch.⁵ Myoinositol is readily taken up by erythrocyte precursors and converted to inositol pentaphosphate (IP5). IP5 and adenosine triphosphate (ATP) concentrations increase rapidly after hatch, with corresponding decreases in blood oxygen affinity due to IP5 binding to hemoglobin. The highest concentration of IP5 is in the mature erythrocyte, and this concentration does not change for the life of the cell regardless of requirements for oxygen. Various degrees of IP5 binding result in a wide range of hemoglobin oxygen affinity among species. However, pH is a much more important regulator of oxygen affinity. Decreasing pH results in decreasing oxygen affinity (Bohr affect), presumably due to IP5 binding.³ Carbon dioxide (CO₂) has a minimal effect on oxygen affinity because the efficient respiratory system of avian species is thought to maintain a very constant blood CO₂ level. Formation of bicarbonate from CO₂ and hydrogen ion release indirectly may regulate oxygen affinity.

GRANULOCYTES

As in other avian species, the peripheral blood of chickens and turkeys contains heterophils, eosinophils, and basophils. The above cells arise from a common precursor in the bone marrow. Maturation of granulocytes is described in Table 122.2.

Heterophils

The heterophil is the most common avian granulocyte. Granules are eosinophilic and rod shaped in chickens and turkeys. However, granules tend to round up in

TABLE 122.2 The Granulocyte Series Maturation Sequence³

Cell Maturation Stage	Description
Myeloblast	Large, round cell with rim of lightly basophilic cytoplasm around a large nucleus with a delicate chromatin pattern and nucleoli
Promyelocyte	Light blue cytoplasm, eccentric nucleus with delicate chromatin pattern, primary granules in cytoplasm are orange spheres, heterophil and basophil promyelocytes also have magenta granules and rings (smaller in basophil promyelocyte)
Myelocyte	Smaller, more condensed nucleus, contain less than half the definitive number of specific or secondary granules and still retain magenta granules and rings (except eosinophil myelocytes)
Metamyelocyte	Smaller, slightly indented nucleus, more than half the number of definitive granules
Band	Similar to mature cell without nuclear lobes; nucleus is elongate to U-shaped, rare to see bands in circulation in avian blood, usually see mature granulocytes only
Mature heterophil	Round cell with no cytoplasmic coloration, eosinophilic rod-shaped cytoplasmic granules with a distinct refractile central body, 2–3 lobed nucleus with coarse chromatin often partially obscured by granules
Mature eosinophil	Round, similar to heterophil but granules more brightly eosinophilic (more arginine) and without central refractile body; cytoplasm usually pale blue
Mature basophil	Round cell with a round central nucleus; deeply basophilic cytoplasmic granules that may partially obscure the nucleus and dissolve or coalesce in alcohol solubilized stains like Wright's

tissue samples due to degeneration. Species variation in granule shape and depth of coloration can occur. The cytoplasm is colorless and the granules commonly cover the nucleus at least partially. The mature heterophil is round and measures approximately 13 μ m in diameter (Figs. 122.2 and 122.3) The nucleus has 2–3 lobes and a coarse chromatin pattern.

Heterophil function is similar to mammalian neutrophil function. However, there are differences in granule contents and response to some stimuli.^{12,13,52,55} It has been speculated that avian heterophils either do not contain or contain minimal quantities of specific lysozyme. Heterophils lack myeloperoxidase and alkaline phosphatase. Chicken and turkey heterophils, unlike neutrophils, do not respond to stimulation by formyl-methionyl-leucylphenylalanine (FMLP) and their production of oxygen radicals is lower than that of mammalian neutrophils.³ However, there is a significant difference in heterophil function when comparing

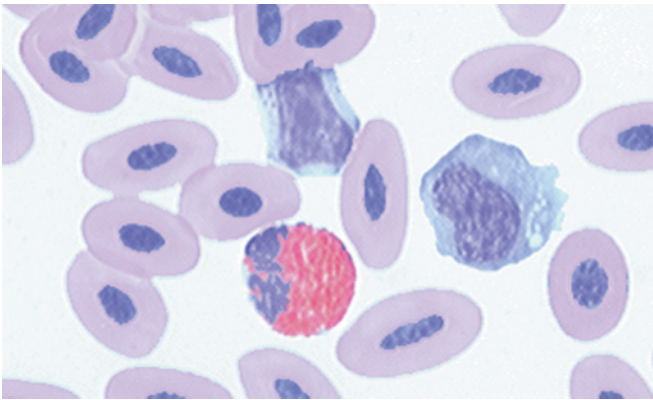


FIGURE 122.2 Medium lymphocyte with intracytoplasmic granules to left of nucleus, monocyte, and heterophil from a wild turkey. Cells are identified in a clockwise manner. Wright-Giemsa stain; magnification 100 \times .

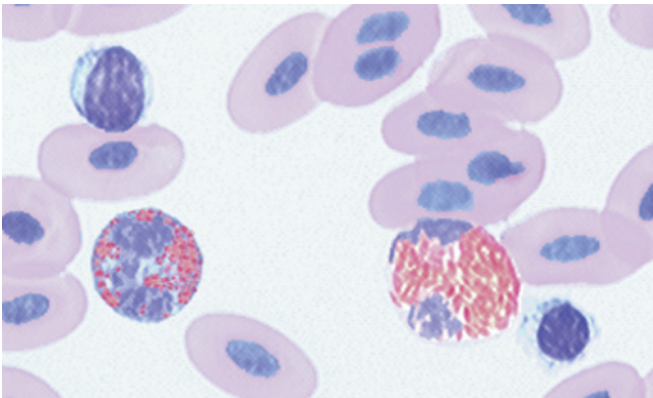


FIGURE 122.3 Lymphocyte, heterophil, thrombocyte, and eosinophil of a domestic chicken. Cells are identified in a clockwise manner. Wright-Giemsa stain; magnification 100 \times .

different lines or strains of chickens and turkeys.^{14,51} Avian heterophils have been shown to participate in antibody dependent cellular cytotoxicity, possess Toll-like receptors, and use extracellular traps to kill antigens.^{7,26} Interleukin-2 (IL-2) is a strong stimulator of neutrophil activation in mammals but is not as strong in chickens. IL-2 stimulation appears to be age dependent with effects noted at 7 and 14 days of age but not at hatch or at 21 days of age. In chickens, IL-2 can modulate certain functions such as priming heterophils for a significantly strengthened respiratory burst subsequent to stimulation with phorbol myristate acetate and increased phagocytosis of opsonized bacteria.²⁷ IL-2 has no effect on degranulation of heterophils post stimulation.

The pineal gland exerts some control of heterophils via the hormone melatonin.⁵³ Heterophils appear to be the first line of defense against bacterial pneumonia, although the ability to generate oxidative metabolites is

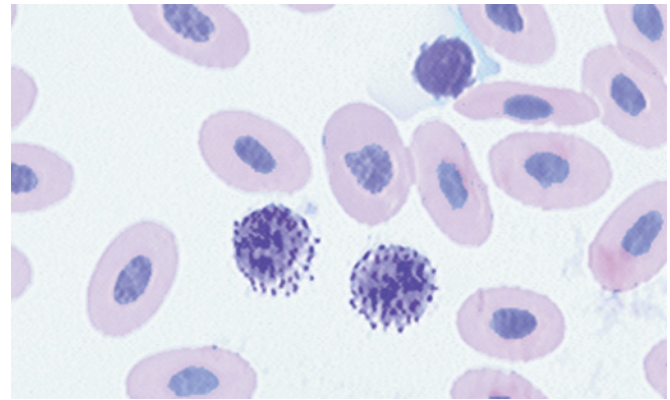


FIGURE 122.4 Thrombocyte and two basophils from a domestic chicken. Cells are identified in a clockwise manner. Wright-Giemsa stain; magnification 100 \times .

lower in heterophils derived from the respiratory tract than in heterophils from the peripheral blood. Defensins are antimicrobial proteins stored in the lysosome-like granules of heterophils, macrophages and epithelial cells. Originally termed “lysosomal cationic proteins” in the neutrophils of rabbits, there are two types, alpha and beta in man, and a third type, theta, found only in Rhesus monkeys. Only the betas have been found so far in birds. Gallinacin-3 has been identified in chickens and gallopavin-1 in turkeys.⁵⁸ The largest (primary) granules contain lysozyme and defensins which are first detectable in the promyelocytic stage adjacent to the Golgi.

Eosinophils

These cells are round to irregularly shaped and are approximately 12 μm in diameter. They have a lobed nucleus and light blue cytoplasm with eosinophilic round to oval granules that stain brighter than those of heterophils and lack the central body (Fig. 122.3). Immature heterophils have round granules and could be confused with eosinophils. Cytochemical stains can distinguish between the two types of cells. Eosinophils are positive for peroxidase and acid phosphatase activity and Sudan black B, while heterophils are negative.³

Basophils

The basophil is a round cell with deeply basophilic granules in the cytoplasm and a round, light blue, central nonindented nucleus. It resembles the mammalian mast cell. Birds appear to have more mast cells and basophils than mammals. The basophil measures approximately 12 μm in diameter and the granules often partially obscure the nucleus (Fig. 122.4). These granules may dissolve or coalesce in Wright's stains. Basophils are one of the first leukocytes to enter tissue as part of the early inflammatory response in birds and the cutaneous basophil response can be measured to help assess immunocompetence.^{3,28}

MONOCYTES

Chicken and turkey monocytes are usually the largest leukocytes (approximately 14µm in diameter) and need to be differentiated from large lymphocytes (Figs. 122.1 and 122.2). Monocytes are round cells, usually have indented nuclei and abundant pale, vacuolated blue-gray cytoplasm. The cytoplasm contains fine azurophilic granules. The monocyte is a pleomorphic cell and can be difficult to identify. It is important to be consistent with lymphocyte classification to aid in differentiation from monocytes. Lymphocytes are round and generally have less cytoplasm than monocytes. Size is dependent upon activation state in chickens and blood monocytes can not be differentiated from lymphocytes solely on the basis of size.

Chicken and turkey monocytes are similar to the mammalian cells. Monocytes from turkeys and chickens are capable of oxidative activity as well as phagocytosis and killing. Heterophils from chickens and turkeys have greater phagocytic activity and killing ability than macrophages.³ More is known about macrophage phagocytosis and killing in the chicken and turkey than about that of monocytes. There are few resident "peritoneal" macrophages harvestable in chickens.^{44,45,55} All macrophages must be elicited, usually using Sephadex™ or other agents. Sephadex™ elicits and activates macrophages via the alternate complement pathway. Macrophages respond to inflammation by induction of adhesion molecules, chemotaxis to the site of greatest concentration of the chemoattractant and, once there, by initiation of a respiratory burst. FMLP is a chemoattractant for chicken macrophages but not for turkey macrophages. Chicken macrophages need higher concentrations of FMLP than human macrophages, possibly due to binding of receptors to different polypeptide structures than their mammalian counterparts. Other chemoattractants to avian macrophages are endotoxin-activated chicken serum and *E. cloacae* culture supernatant. The respiratory burst consumes oxygen for the production of superoxide anion, hydrogen peroxide and eventually hydroxyl radical and singlet oxygen.^{44,45} Early inflammatory macrophages from young chickens are better than blood monocytes or late inflammatory macrophages for generation of superoxide anion after stimulation with phorbol myristic acid. Macrophages kill tumor cells by four different processes: (1) via tumor necrosis factor, (2) via antibody dependent cellular cytotoxicity, (3) via an arginine dependent process and (4) via direct contact.⁵⁵ Since birds are uricotelic, there is no *de novo* synthesis of L-arginine as in the mammalian urea cycle, and this amino acid is required in birds for the production of these intermediates.³ The arginine dependent process probably uses the enzyme arginine deiminase (in some papers referred to as arginase) to convert arginine to citrulline and nitric oxide (NO) which have been associated with the ability of macrophages to deplete tumor cells of iron-sulfur prosthetic groups required for mitochondrial respiration. Another pathway to produce NO is via the enzyme inducible

TABLE 122.3 The Monocyte Maturation Sequence³

Cell Maturation Stage	Description
Monoblast	A poorly defined cell, probably indistinguishable from the myeloblast
Early promonocyte	Large cells with abundant, clear blue cytoplasm, round nucleus, reticular chromatin
Late promonocyte	Round, eccentric nucleus with granular basophilic cytoplasm, sparse eosinophilic granules
Mature monocyte	Large and irregular with a round to bilobed nucleus, fine chromatin pattern, abundant blue-gray granular cytoplasm, and occasional cytoplasmic vacuoles and/or fine dust-like eosinophilic granules

NO synthase in place of arginine deiminase. The activation state and the phenotype of the pathogen are important factors in the macrophage's ability to kill a pathogen. The monocyte maturation series is described in Table 122.3.

LYMPHOCYTES

Lymphocytes are the predominant leukocyte in the peripheral blood of chickens and turkeys. Both small and medium lymphocytes normally occur. The small lymphocytes are round with a round nucleus, clumped chromatin, high nuclear:cytoplasmic (N:C) ratio, and a rim or small amount of basophilic cytoplasm (Figs. 122.1 and 122.3). Occasionally the cytoplasm of small lymphocytes may only be seen as cytoplasmic projections, and they may be confused with thrombocytes. Thrombocytes can be distinguished by their clear cytoplasm. Medium lymphocytes have more abundant and sometimes more pale basophilic cytoplasm. They often "mold" around adjacent cells. Lymphocytes may have nuclear indentation, and sometimes larger lymphocytes may have more angular nuclei or nuclei with a flattened side. Medium to large lymphocytes must be distinguished from monocytes as mentioned previously.

Reactive lymphocytes can be seen in peripheral blood. Cell size is increased and the cytoplasm is deeply basophilic, sometimes with a clear perinuclear area (Golgi). Plasma cells may only rarely be found in peripheral blood. Reactive lymphocytes may resemble rubricytes. However, rubricytes have a lower N:C ratio and are typically seen in peripheral blood with polychromatophilic erythrocytes in regenerative responses.³ The presence of eosinophilic (azurophilic) granules in peripheral blood lymphocytes is rare, and its significance is unknown (Fig. 122.2). These cells are readily distinguished from monocytes, because the lymphocyte azurophilic granules are larger and more intensely staining than the dust-like granules of monocytes.

TABLE 122.4 The Thrombocyte Maturation Sequence³

Cell Maturation Stage	Description
Thromboplast	Large, ameboid cells with a rim of very basophilic cytoplasm around a large nucleus with punctate chromatin (chromatin is not coarse as in the rubriblast), nucleolus not always apparent, some clear vacuoles within cytoplasm
Early immature thrombocyte	Slightly lower N:C than thromboplast, more cytoplasmic vacuoles evident; chromatin becomes clumped

THROMBOCYTES

Chicken and turkey thrombocytes are round to slightly oval cells with a round nucleus in the center of a clear cytoplasm. Thrombocytes can be confused with small lymphocytes in tissue samples and blood smears as mentioned previously. Monoclonal antibodies against chicken thrombocytes have been developed and can aid in identification.²¹ Avian thrombocytes contain azurophilic cytoplasmic granules, stain periodic acid-Schiff (PAS) and Grimelius positive, clump readily and produce little thromboplastin. It is likely that they are capable of phagocytosis, although definitive proof has not been obtained.³¹ Thrombocytes respond to lipopolysaccharide through Toll-like receptor 4.⁵⁰ Frequently, one or more distinct small granules are present at the poles of the thrombocyte (Fig. 122.1). A C3b-like receptor and analogs to mammalian platelet glycoproteins IIb and IIIa have been identified in chicken thrombocytes.³ Nuclear retention in avian thrombocytes results in the continued ability of the mature cell to synthesize proteins. Similar to mammalian platelets, avian thrombocytes have fibrinogen receptors that localize when thrombocytes are activated, facilitating thrombocyte aggregation.³ The thrombocyte maturation series is described in Table 122.4.

NORMAL REFERENCE INTERVALS FOR CHICKENS AND TURKEYS

Total erythrocyte numbers are approximately $3 \times 10^6/\mu\text{L}$, or a PCV of 25–42%. Chicks typically have a lower PCV (as low as 24%) that increases with age. The MCV has been reported in the range 90–140 fL.³ Chicks tend to have fewer, larger erythrocytes. Hemoglobin concentrations of chickens and turkeys have been reported from 8.6 to 15.2 gm/dL.³ Variation in erythrocyte parameters may reflect different lines of chickens and turkeys and different methods of determination. Erythrocyte lifespan is 28–35 days in chickens.³

Early data showed PCV to be lower in heavy type (30.1–30.9%) than in White Leghorn type (31.9–33.9%) chickens.³ More recent hematology data of chickens are

TABLE 122.5 Hematologic Values for the Chicken (*Gallus gallus domesticus*)³

Parameter	Interval
Erythrocytes (μL)	2,500,000–3,500,000
Hemoglobin (g/dL)	7–13
PCV (%)	22–35
MCV (fL)	90–140
MCH (pg)	33–47
MCHC (%)	26–35
Reticulocytes (%)	0–0.6
Leukocytes (μL)	12,000–30,000
Heterophils (band)	Rare
Heterophils	3000–6000
Lymphocytes	7000–17,500
Monocytes	150–2000
Eosinophils	0–1000
Basophils	Rare

TABLE 122.6 Reference Intervals for Leukocytes From Healthy 22–24 Week Old Male Broiler-Type Chickens ($N = 89$)³

Cell Type	Reference Interval
Leukocytes (μL)	7940–24,280
Heterophils	1703–9746
Lymphocytes	2639–10,294
Monocytes	544–4123
Eosinophils	0–346
Basophils	382–2499

TABLE 122.7 Leukocyte Values From 10-Day-Old Broiler Breeder Chicks ($N = 10$)³

Cell Type	Values
Leukocytes (μL)	25.19 ± 5.01
Heterophils	7.19 ± 3.39
Lymphocytes	13.86 ± 1.58
Monocytes	1.94 ± 1.01
Eosinophils	0.64 ± 0.92
Basophils	1.56 ± 0.25

shown in Tables 122.5, 122.6, and 122.7. Mean hematocrits from specific pathogen-free White Leghorn chicks of 5–42 days of age ranged from 32.7% to 36.7%, respectively.³

Limited hematologic data are available for domestic turkeys (Table 122.8). Reference intervals for 4 month old wild turkeys are detailed in Table 122.9. As in chickens, studies have shown that differences exist in hematologic parameters of closed genetic lines and different

TABLE 122.8 Hematologic Values for 3-Week-Old Domestic Turkeys (N = 24)³

Parameter	Value
Hemoglobin (g/dL)	9.14
Hematocrit (%)	30.8
MCV (μm)	164
MCH (pg)	42.6
MCHC (%)	29.6

TABLE 122.9 Reference Intervals for Healthy Wild Turkey Poults (*Meleagris gallopova* subsp. *silvestris*) (N = 48)³

Parameter	Reference Interval
PCV (%)	30–41.5
Leukocytes (/μL)	13,917–46,609
Heterophils	4046–24,231
Lymphocytes	4156–31,138
Monocytes	0–3756
Eosinophils	0–420
Basophils	23–2039

commercial lines of turkeys.^{14,22,51} Large body birds bred for meat production had lower lymphocyte counts, higher heterophil counts, and higher total erythrocyte counts than lines bred for egg production.³

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Hematology of Psittacines

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Acronyms and Abbreviations

EDTA, ethylenediaminetetraacetic acid; Hgb, hemoglobin; H:L, heterophil:lymphocyte ratio; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; M:E, myeloid:erythroid ratio; N:C, nuclear:cytoplasmic ratio; PCV, packed cell volume; RBC, red blood cell; WBC, white blood cell.

ERYTHROCYTE MORPHOLOGY

The normal mature psittacine red blood cell (RBC) is a large (compared to mammalian RBC), flattened elliptical cell with an elliptical, centrally positioned nucleus and smooth nuclear margins (Fig. 123.1). The nuclear chromatin is uniformly clumped and becomes increasingly condensed with cellular age. In Wright-stained blood films, the nucleus stains purple while the cytoplasm appears orange-pink with a uniform texture.

The shape of RBCs in blood films from normal psittacine birds is relatively uniform with slight variability; therefore, a minor degree of poikilocytosis is considered to be normal. Variations in RBC color include polychromasia and hypochromasia. Polychromatophilic RBCs occur in low numbers (usually less than 5% of the total RBC count) in the blood of most normal birds. Polychromatophilic RBCs are similar in size to mature RBCs and appear as reticulocytes when stained with vital stains, such as new methylene blue. The cytoplasm appears weakly basophilic, and the nucleus is less condensed than the nucleus of mature RBCs.

Determination of reticulocyte count can be made by staining RBCs with a vital stain, such as new methylene blue. Reticulocytes have a distinct ring of aggregated

reticular material that encircles the nucleus. As the cells mature the amount of aggregated reticular material decreases and becomes more dispersed throughout the cytoplasm. With further maturation the reticular material becomes nonaggregated, and the cells begin to resemble the “punctate” reticulocytes of felids; therefore, most mature avian RBCs contain a varying amount of aggregate or punctate reticulum. Reticulocytes that reflect the current RBC regenerative response are those that have a distinct ring of aggregated reticulum that encircles the RBC nucleus.^{1,9}

Hematologic methods used to evaluate avian erythrocytes include determination of a packed cell volume (PCV) or microhematocrit, hemoglobin (Hgb) concentration, RBC count, and evaluation of the RBC morphology on a stained blood film. The PCV is determined using the same method used in mammalian hematology. The hemoglobin concentration is determined using the cyanmethemoglobin method with centrifugation of the free nuclei from lysed erythrocytes before obtaining the optical density value to avoid overestimation of the true Hgb concentration. A RBC count can be determined by using automated methods (electronic particle counter) or manual methods. Two manual methods commonly used are the erythrocyte Unopette (Unopette,

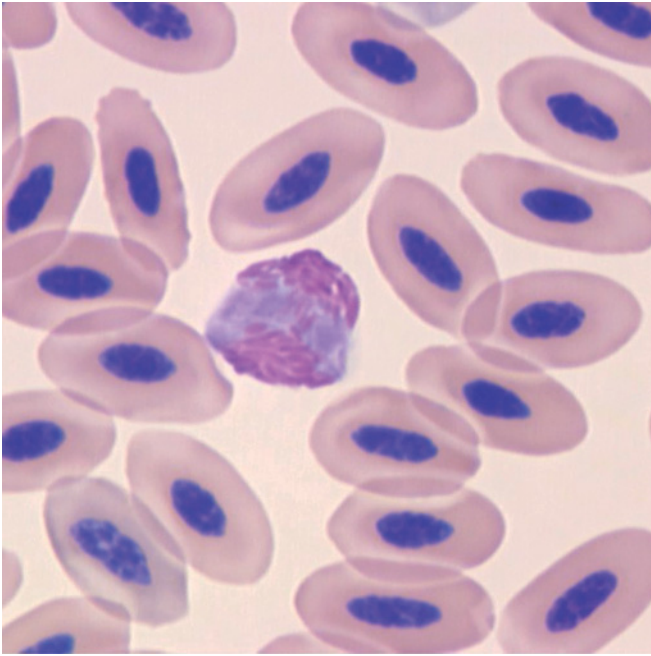


FIGURE 123.1 Blood film of a parrot. Normal mature RBCs and a heterophil. Wright stain.

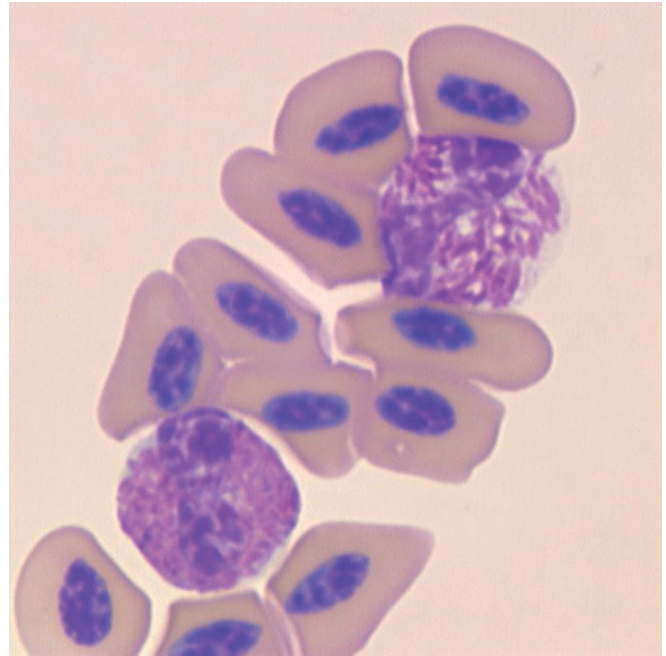


FIGURE 123.2 Blood film of a parrot. A heterophil (top) and an eosinophil (bottom). Wright stain.

Becton-Dickinson, Rutherford, NJ) method as used in mammalian hematology and the Natt and Herrick method.¹ Both methods are labor intensive and require accurate dilutions (made easier using the Unopette system) and counting cells in a hemacytometer. Once the PCV, Hgb concentration, and total RBC count have been determined, the secondary hematologic indices, including the mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), and mean corpuscular hemoglobin (MCH) can be determined.

LEUKOCYTE MORPHOLOGY

Leukocytes in the blood of a psittacine bird include lymphocytes, monocytes, and granulocytes. The granulocytes are further classified as heterophils, eosinophils, and basophils. In general, psittacine heterophils tend to be round (although their shape may be distorted) and uniform in size. The nucleus of the mature heterophil, which is frequently partially hidden by the cytoplasmic granules, is lobed (two to three lobes), with coarse, clumped, purple-staining chromatin (Figs. 123.1, 123.2, and 123.3). The cytoplasm of normal mature heterophils appears colorless and contains granules that stain an eosinophilic color (dark orange to brown red) with Romanowsky-type stains, although the granules can be affected by the staining process and may appear atypical (i.e. poorly stained, partially dissolved, or fused). Typically the cytoplasmic granules of psittacine heterophils appear elongated (rod or spicular shaped) and frequently contain a central refractile body.

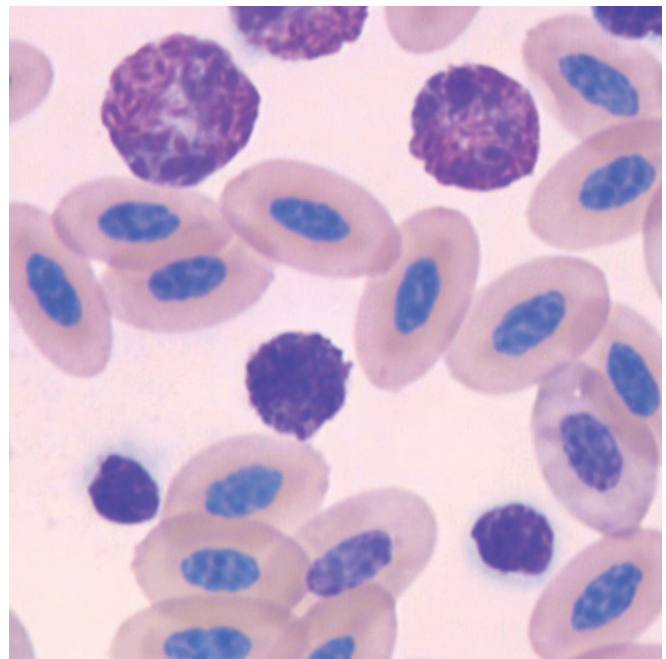


FIGURE 123.3 Blood film of a parrot. Two heterophils (top), a basophil (middle), and two thrombocytes (bottom). Wright stain.

In general, most eosinophils of psittacine birds are similar in size to the heterophils in the same blood film. The nucleus of the eosinophil is lobed and usually stains darker than a heterophil nucleus and the cytoplasm stains clear blue in contrast to the colorless cyto-

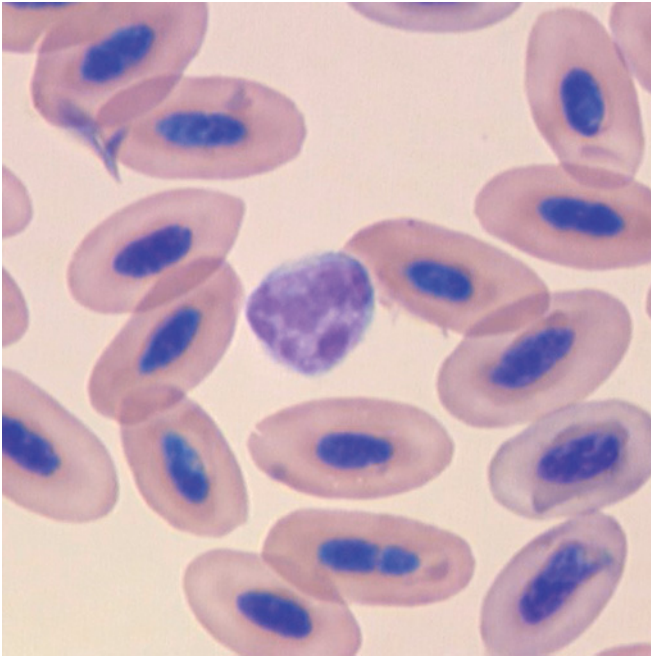


FIGURE 123.4 Blood film of a parrot. A small mature lymphocyte. Wright stain.

plasm of normal mature heterophils (Fig. 123.2). The cytoplasmic granules of these cells are strongly eosinophilic and are typically round in shape and tend to stain more intensely compared to granules of heterophils in the same Romanowsky-stained blood film. Occasionally, granules of eosinophils of psittacine birds may be affected by the staining process and appear colorless, pale blue, or swollen.

Psittacine basophils contain deeply metachromic granules that frequently obscure the cell nucleus (Fig. 123.3). The nucleus is usually nonlobed, causing them to resemble mammalian mast cells. The cytoplasmic granules of basophils are frequently affected by alcohol-solubilized stains and may partially dissolve or coalesce; therefore, they may appear abnormal in blood films stained with Romanowsky-type stains. Unlike mammals, basophils are frequently found in the peripheral blood of psittacine birds.

Avian lymphocytes resemble mammalian lymphocytes (Fig. 123.4). They are mononuclear cells identified as round cells that frequently show cytoplasmic irregularity as they mold around adjacent RBCs in the blood film. Lymphocytes have a round, occasionally slightly indented, centrally or slightly eccentrically positioned nucleus. The nuclear chromatin is heavily clumped or reticulated in mature lymphocytes. The cytoplasm of small mature lymphocytes is typically scant resulting in a high nuclear to cytoplasm ratio (N:C), and typically appears homogeneous, weakly basophilic (pale blue), and lacks vacuoles or granules. Although small mature lymphocytes are the most frequent form of lymphocyte in blood films; medium and large lymphocytes with more abundant and lighter

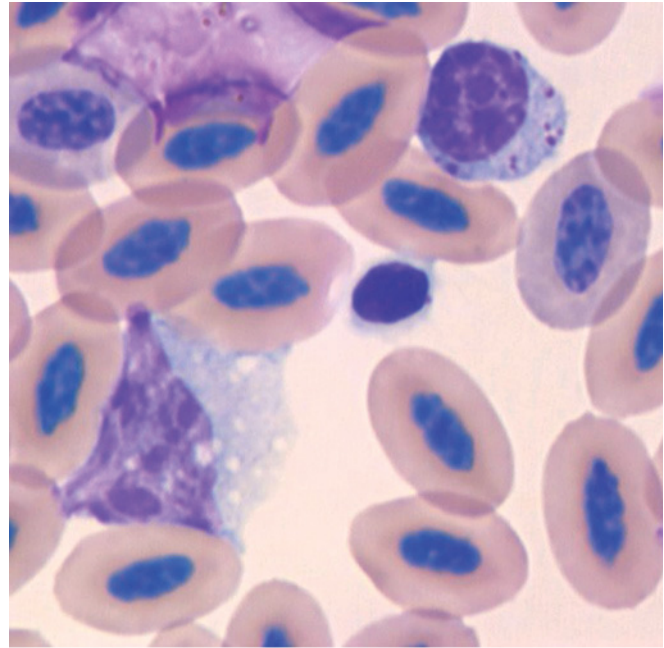


FIGURE 123.5 Blood film of a parrot. A monocyte (bottom) and a large lymphocyte with azurophilic granules (top right). Wright stain.

staining cytoplasm may occur as well. Occasionally, lymphocytes in the blood film of normal psittacine birds may contain distinct azurophilic granules or irregular cytoplasmic projections (Fig. 123.5).

Monocytes in the blood films from normal psittacine birds are usually the largest leukocytes and resemble their mammalian counterpart. These cells vary in shape from round to amoeboid (Fig. 123.5). The nucleus of monocytes also varies in shape and can appear round to lobed with relatively pale, less dense chromatin as compared to lymphocyte nuclei. The cytoplasm of the psittacine monocyte is abundant, blue-gray in color and may appear slightly opaque. Vacuoles or fine dust-like eosinophilic granules may also be present.

An avian total white blood cell (WBC) count is obtained by most laboratories using a manual method. The two commonly used manual methods are the direct and the semidirect methods. The semidirect method involves the staining of avian heterophils and eosinophils with a phloxine B solution that is also used as a diluent. This method uses a hemacytometer to count cells (heterophils and eosinophils) that stain red to obtain an absolute heterophil and eosinophil count. Because other leukocytes are not counted, they must be factored into the total leukocyte count after obtaining a leukocyte differential from a stained blood film. An absolute heterophil and eosinophil count is obtained with the appropriate formula using the dilution factor and number of squares counted as one would use for counting cells in a hemacytometer. Once this has been determined, the total WBC count is determined by multiplying the absolute heterophil and eosinophil count (heterophils and eosinophils/mm³) by 100 and dividing

that number by the percent heterophils and eosinophils from the leukocyte differential.

The commonly used direct manual method for obtaining a total leukocyte count involves making a 1:200 dilution of the blood with Natt and Herrick solution to charge the hemacytometer. The total leukocyte count is obtained by counting the dark blue cells (presumed to be leukocytes) in the hemacytometer. The advantage of this method is that an absolute leukocyte, erythrocyte, and thrombocyte count can be obtained from the same charged hemacytometer. The disadvantage is that it is often difficult to differentiate small lymphocytes from thrombocytes.

THROMBOCYTE MORPHOLOGY

Like all avian thrombocytes, those in the blood films of psittacine birds are nucleated cells and are the second most numerous cell (after RBCs) found in the blood. Thrombocytes are typically small round to oval cells (smaller than RBCs) with a round to oval nucleus that contains densely clumped chromatin (Fig. 123.3). The nucleus is more round than an RBC nucleus and thrombocytes tend to have a higher N:C ratio. The cytoplasm of normal mature thrombocytes is colorless to pale gray and may have a reticulated appearance. This feature is useful in differentiating thrombocytes from small mature lymphocytes. Thrombocytes also frequently contain one or more distinct eosinophilic (specific) granules (actually aggregates of many small granules) grouped in one area of the cytoplasm.

Absolute thrombocyte counts are rarely determined in avian blood samples because they tend to clump, making meaningful counts difficult. The direct manual method for obtaining a total thrombocyte count involves making a 1:200 dilution of the blood with Natt and Herrick's solution to charge the hemacytometer. The total thrombocyte count is obtained by counting the small oval cells that fail to stain blue in the hemacytometer. Alternatively, determination of the number of thrombocytes in relation to the number of erythrocytes on the blood film provides a reasonable assessment of the thrombocyte numbers. In general, 10–15 thrombocytes per 1000 erythrocytes are considered to be an adequate number for normal birds. Numbers less than that suggest thrombocytopenia and those greater suggest thrombocytosis.

REFERENCE INTERVALS

Age Influences

Little has been reported in the literature concerning the influence of aging on the hemogram of psittacine birds. In one study, the PCV and MCV tended to increase with age in budgerigars (*Melopsittacus undulates*), whereas the MCHC decreased.⁸ Because the Hgb was unaffected by aging, it was presumed that the decline in MCHC

was a function of an increase in MCV. No age-related changes in the leukogram were detected in this study.

A transition from a differential WBC count that is primarily heterophilic to one that is primarily lymphocytic has been observed in several species of psittacine birds as they mature; however, the opposite has been reported in other species.⁶ It is speculated that this change is species-specific.

In another budgerigar study, male birds had higher RBC counts, PCV values, and Hgb concentrations compared to females.¹⁰ The reason for this may be related to the negative influence of estrogen on erythropoiesis in females and the positive influence of testosterone in males. In other studies no differences in the hemogram were noted between males and females; however, it should be noted that frequently the gender of a psittacine bird is unknown.

Species Influences

Normal reference intervals (Table 123.1) reveal that with a few exceptions, psittacine birds have similar RBC parameters: PCV of 35–55%; RBC count of 2.4–5.0; hemoglobin concentration of 11–16 mg/dL; MCV of 90–200 fL; and MCHC of 22–33%.¹ Likewise, the normal total WBC count of most psittacine birds tends to fall within a range 5,000–15,000/ μ L.

Differences in the normal heterophil: lymphocyte (H:L) ratios of various psittacine birds have been reported. For example, evaluation of the data of three species of macaws in one study revealed an H:L ratio of 2.1–2.2, whereas another study reported the range between 0.7 and 0.9.^{11,16} In general, most psittacine birds appear to exhibit a predominance of heterophils with an (H:L) ratio ranging between 1.1 and 2.4 (average 1.7) when examining reported reference intervals.^{3,4}

Physiological and Environmental Influences

Reference intervals for hematologic parameters of psittacine birds have a broad range owing to the influence of various intrinsic and extrinsic factors. In general, avian hematological values are subject to extensive variability resulting from different environment and management practices that can affect physiological responses.⁷ For example, heterophil numbers are altered by seasonal changes, diurnal rhythm, gender, age, and diet.^{5,6,14,15,17} Normal hematologic values vary between the species as well. The different avian species from which blood samples are submitted to veterinary laboratories creates a significant logistical challenge to the development of clinically relevant normal values. Thus, published reference intervals should only be used as guidelines.

Blood Sampling Conditions

Blood collection for hematologic evaluation in psittacine birds begins with proper restraint and many birds

TABLE 123.1 Hematology Values for Selected Captive Psittacine Birds (Mean ± S.D. and Range)^a

	White Cockatoo (<i>Cacatua alba</i>)	Red Lory (<i>Eos bornea</i>)	Blue-fronted Amazon (<i>Amazona aestiva</i>)	Orange-winged Amazon (<i>Amazona amazonica</i>)	Scarlet Macaw (<i>Ara macao</i>)	Green-winged Macaw (<i>Ara chloroptera</i>)	Military Macaw (<i>Ara militaris</i>)	Blue and Gold Macaw (<i>Ara ararauna</i>)	Patagonian Conure (<i>Cyanoliseu spatagonus</i>)	African Gray Parrot (<i>Psittacus erithacus</i>)
RBC (×10 ⁶ /μL)	2.98 ± 0.19 (2.75–3.2)	3.17 ± 0.49 (2.62–4.72)	2.92 ± 0.55 (2.11–3.53)	3.08 ± 0.20 (2.81–3.32)	3.07 ± 0.43 (2.29–3.67)	3.20 ± 0.38 (2.65–4.05)	3.44 ± 0.59 (2.72–5.16)	3.24 ± 0.50 (2.11–4.10)	3.54 ± 0.32 (3.16–4.09)	3.47 ± 0.41 (2.96–4.03)
PCV (%)	44.8 ± 4.4 (37.0–48.0)	48.8 ± 3.2 (44.0–54.0)	50.6 ± 6.2 (43.5–58.0)	48.4 ± 2.2 (46.0–51.0)	47.4 ± 4.4 (40.0–54.0)	46.1 ± 3.8 (39.0–54.0)	47.1 ± 4.3 (37.0–54.5)	44.6 ± 4.6 (31.5–51.8)	47.6 ± 3.1 (45.0–52.0)	46.4 ± 5.3 (32.0–54.0)
Hb (g/dL)	15.7 ± 1.9 (13.9–18.4)	16.0 ± 1.4 (14.2–18.7)	17.3 ± 0.9 (16.0–18.4)	16.3 ± 0.8 (15.5–17.5)	16.4 ± 2.2 (13.1–19.9)	13.6 ± 2.8 (9.6–18.7)	15.6 ± 2.3 (11.1–19.6)	14.4 ± 1.2 (11.7–17.0)	15.0 ± 0.8 (14.3–16.2)	15.4 ± 3.4 (10.7–21.7)
MCV (fL)	150.7 ± 14.3 (132–171)	155.9 ± 13.7 (111–172)	180.0 ± 19.7 (163–209)	157.6 ± 5.9 (151–166)	151.6 ± 10.7 (135–169)	145.0 ± 13.9 (116–177)	137.0 ± 16.0 (106–173)	141.0 ± 21.4 (102–199)	134.6 ± 7.1 (127–146)	137.7 ± 17.1 (106–166)
MCHC (g/dL)	33.5 ± 4.0 (30.0–39.2)	32.9 ± 1.0 (31.2–35.6)	34.3 ± 2.6 (31.7–37.8)	33.7 ± 1.5 (32.1–36.0)	34.4 ± 2.2 (29.7–37.3)	29.6 ± 4.6 (21.9–34.9)	33.5 ± 2.5 (33.9–40.7)	32.7 ± 3.4 (28.1–43.5)	31.6 ± 0.5 (30.9–32.3)	32.2 ± 5.4 (24.8–43.4)
WBC (×10 ³ /μL)	6.7 ± 7.5 (1.3–18.7)	3.3 ± 2.2 (0.8–9.0)	6.5 ± 2.4 (4.7–11.0)	6.1 ± 3.8 (1.2–10.1)	9.8 ± 4.5 (4.7–22.0)	16.9 ± 8.9 (3.8–30.0)	9.5 ± 4.5 (13.7–18.0)	16.6 ± 9.0 (1.7–36.0)	5.8 ± 2.1 (2.5–8.7)	9.0 ± 3.6 (29.4–83.0)
Heterophils (%)	45.1 ± 28.5 (17.6–83.0)	55.2 ± 17.4 (25.6–79.2)	30.7 ± 15.0 (12.4–46.6)	36.2 ± 7.2 (21.9–40.7)	39.9 ± 13.0 (26.0–67.0)	32.2 ± 13.4 (14.0–62.0)	41.5 ± 15.4 (12.0–62.5)	37.2 ± 18.3 (12.8–60.0)	40.7 ± 13.7 (23.5–62.7)	60.8 ± 20.6 (29.4–83.0)
Lymphocytes (%)	52.7 ± 27.5 (15.0–80.3)	41.7 ± 16.6 (18.7–70.1)	67.0 ± 14.2 (52.4–83.5)	63.4 ± 7.0 (55.8–73.2)	55.1 ± 11.4 (36.0–68.2)	34.0 ± 13.8 (35.0–84.2)	55.3 ± 14.5 (43.3–80.0)	60.0 ± 17.6 (35.5–84.4)	54.3 ± 10.7 (34.7–65.8)	35.5 ± 20.9 (15.5–67.7)
Monocytes (%)	1.8 ± 1.6 (0.0–3.7)	1.4 ± 1.2 (0.0–4.5)	1.7 ± 0.9 (1.0–3.1)	3.5 ± 1.5 (2.0–5.0)	3.4 ± 2.4 (0.0–8.1)	2.1 ± 2.2 (0.0–8.3)	2.4 ± 2.4 (0.0–8.0)	1.3 ± 0.8 (0.0–2.0)	0.9 ± 1.1 (0.0–2.5)	2.8 ± 2.0 (1.0–6.0)
Eosinophils (%)	0.2 ± 0.4 (0.0–1.0)	1.5 ± 1.5 (0.0–4.6)	0.3 ± 0.5 (0.0–1.0)	1.0 ± 2.2 (0.0–5.0)	1.2 ± 1.4 (0.0–4.0)	0.5 ± 0.9 (0.0–3.0)	0.3 ± 0.7 (0.2–2.1)	0.7 ± 0.8 (0.0–2.0)	0.2 ± 0.4 (0.0–1.1)	1.0 ± 1.2 (0.0–2.8)
Basophils (%)	0.2 ± 0.4 (0.0–1.0)	0.2 ± 0.4 (0.0–1.2)	0.2 ± 0.5 (0.0–1.0)	0.3 ± 0.7 (0.0–1.7)	0.4 ± 0.7 (0.0–2.0)	0.3 ± 0.5 (0.0–1.7)	0.2 ± 0.4 (0.0–1.2)	0.3 ± 0.6 (0.0–1.2)	0.0	0.0

^aAdapted from Polo FJ, Peinado VI, Viscor G, et al. Hematologic and plasma chemistry values in captive psittacine birds. *Avian Dis* 1998;42:523–535.

can be physically restrained without anesthesia. The beak of these birds can inflict injury to the handler; therefore, proper restraint of the head is required. Cloth towels can be used to safely restrain psittacine birds during the blood collection procedure as long as excessive restraint around the keel is avoided to prevent asphyxiation. Excessive stress that occurs during capture and restraint of birds, especially those not accustomed to being handled or that are ill, can further compromise the health of the patient and alter the hematologic indices.

The blood volume of the galah (*Eolophus roseicapillus*), using the indocyanine green method was determined to be $10.6 \pm 2.9\%$ of body weight; therefore, the blood volume of psittacine birds is generally considered to be 10% of the body weight.¹² A maximum blood sample representing one percent of the bird's body weight (or 10% of the blood volume) is generally considered to be a safe amount of blood that can be obtained from a psittacine bird. For routine hematological evaluations in birds, a sample size of 0.2mL is usually adequate.

A variety of venipuncture sites for blood collection in psittacine birds are available and include the jugular, basilic vein (cutaneous ulnar, wing or brachial), and the medial metatarsal (caudal tibial) vein. Blood can be collected using a needle and syringe when performing venipuncture on the jugular vein or other large veins. Collection of blood with vacuum tubes is not recommended because the excessive aspiration pressure that occurs causes the thin walled veins to collapse during the procedure. A needle and syringe without the use of an anticoagulant is commonly used with rapid collection and transfer of the sample into a collection tube containing the appropriate anticoagulant, such as ethylenediaminetetraacetic acid (EDTA). This method allows for the blood sample to be divided into various collection tubes depending on the intended use of the sample. Blood samples may also be obtained using an anticoagulant in the syringe at the time of collection. A disadvantage of this technique is that the amount of blood collected is not always predictable and frequently results in an inappropriate ratio of anticoagulant to blood.

In general, a short (1 inch [2.54cm] or less), 25–22 gauge needle attached to a 3mL syringe is typically used for jugular venipuncture. Venipuncture of the right jugular vein can be performed in most psittacine birds and is the blood collection method of choice for small birds that lack other veins large enough for venipuncture. Because this vein is highly movable and surrounded by a large subcutaneous space predisposing this method of blood collection to hematoma formation, the jugular vein must be stabilized before attempting venipuncture.

The basilic vein crosses the ventral surface of the humeral-radioulnar joint (elbow) directly beneath the skin and is easily visualized by wetting the area lightly with alcohol once the bird is restrained and in dorsal recumbency with the wing stretched away from the body. Venipuncture of this vein can be used to collect

blood from medium to large psittacine birds; however, unless anesthetized, most will struggle and move during this type of restraint making blood collection without laceration of the vein difficult.

Venipuncture of the medial metatarsal vein is a common blood collection method in psittacine birds. Blood can be collected either by aspiration into a syringe or by using the drip technique that allows the blood to flow from a needle into a collection tube. The medial metatarsal vein is located on the caudomedial aspect of the tibiotarsus just above the tibiotarsus-tarsometatarsal joint. The vein frequently courses along the caudal aspect of the tibiotarsus and often hides beneath the calcaneal tendon. Because the leg is easy to restrain and the vein is protected by the surrounding muscles of the leg, hematoma formation is minimal compared to venipuncture of a basilic or jugular veins.

BONE MARROW SAMPLING

Collection Sites

Bone marrow samples for cytological evaluation can be successfully obtained in psittacine birds by aspiration of the proximal tibiotarsus.¹ Marrow may also be collected from most of the long bones, except the pneumatic bones. A general or local anesthetic can be used. The type of biopsy needle used for aspiration depends upon the size of the bird, location of the biopsy site, and preference of the cytologist. Bone marrow biopsy needles frequently used for marrow collection in domestic mammals and humans (Jamshidi bone marrow biopsy-aspiration needles and disposable Jamshidi Illinois-Sternal/Iliac aspiration needles, Kormed Corp, Minneapolis, MN) can be used for marrow collection in birds. The pediatric sizes are preferred because of the relatively small bone size of most birds compared to mammals. Spinal needles containing a stylet can be used for marrow collection in very small birds.

The procedure for collecting bone marrow from the proximal tibiotarsus begins with application of a skin disinfectant as for any surgical procedure. The medial or cranial aspect of the proximal tibiotarsus just below the femoral-tibiotarsal joint is a suitable location for aspiration. A small skin incision is made using a scalpel blade to facilitate passage of the needle through the skin. The biopsy needle with stylet is placed against the bone and using gentle pressure and rotary movements, the needle is advanced into the marrow cavity. A perpendicular approach to the bone should be used. The hand not used for manipulating the needle is used to stabilize the tibiotarsus. Once the needle is positioned into the marrow cavity, the stylet is removed and a 3–6mL syringe is attached. The marrow sample is aspirated only into the lumen of the needle by applying negative pressure to the syringe. Excessive or prolonged negative pressure should be avoided to minimize blood contamination of the marrow sample.

When aspiration is completed, the needle and syringe are removed from the tibiotarsus, making sure negative

pressure is not being applied to the syringe. The needle is removed from the syringe, the syringe is filled with air, and the marrow is forced from the needle lumen onto a glass microscope slide. A second glass microscope slide is placed on top of the marrow sample and the marrow is allowed to spread between the two slides as they are pulled apart. Marrow core biopsies for histologic evaluation can be obtained from birds using a technique similar to marrow aspiration. Once the bone marrow biopsy needle is introduced into the marrow space, the stylet is removed and the needle is advanced deeper into the marrow cavity toward the opposite cortex. Once the opposite cortex has been reached, the needle is twisted and redirected slightly to detach the marrow plug within the lumen of the needle. Gentle vacuum may be applied to the syringe to aid in holding the marrow plug in the needle as the needle is withdrawn from the marrow cavity. The marrow core sample is removed by reinsertion of the stylet (usually beginning at the tip of the needle) to push the sample out of the needle. Imprint films can be made from the core sample for cytological evaluation before the sample is placed in neutral buffered 10% formalin. A sample holder is frequently required to maintain the marrow core while it is being fixed in the formalin solution.

Bone marrow slides are stained with the same Romanowsky stains used for staining blood films. Interpretation of the avian bone marrow begins with scanning the marrow film, using the 10× microscope objective to evaluate the number and distribution of cells. Because an actual cell count of a bone marrow sample cannot be obtained, the cellularity is estimated by evaluation of the ratio of fat and cells in marrow particles and compared with the cellularity of normal bone marrow. Normal bone marrow films should provide enough hematopoietic cells to easily perform a 500-cell differential. The degree of cellularity is estimated as poor, normal, or high.

The distribution of cells can also be estimated. Myeloid, erythroid, and thrombocytic elements may appear normal, decreased, or increased. While a more objective approach is to perform an actual differential count based on 1000 cells or more, this is time consuming, and may not provide more information.

Besides estimating the degree of cellularity and evaluating the distribution of the types of cells in the marrow sample, the cytologist should also estimate the myeloid:erythroid ratio (M:E). Any changes involving the maturation sequence of each cell line should be noted. The cell lines include erythroid cells, granulocytes (heterophils, eosinophils, and basophils), monocytes, and thrombocytes. Other cells occasionally found include lymphocytes, plasma cells, osteoblasts, and osteoclasts. The presence of abnormal cells should also be noted.

An accurate interpretation of the bone marrow response can be made only in conjunction with knowledge of the current cellular changes in the blood. Therefore, an evaluation of a hemogram should be made from a blood sample collected at the same time the bone marrow sample is obtained.

Cell Morphology

Erythropoiesis

The stages of maturation in normal avian erythropoiesis appear to be similar to those of mammals. The terminology used for the different stages of erythrocyte maturation varies in the literature.^{1,9,13} In general, there are six recognizable stages involved in RBC development based upon Romanowsky stains. These include rubriblasts, prorubricytes, basophilic rubricytes, early polychromatic rubricytes, late polychromatic rubricytes, polychromatic RBCs, and mature RBCs. As erythroid cells mature, the nuclear size decreases, the chromatin becomes increasingly condensed, the nuclear shape changes from round to ellipsoid, the amount of cytoplasm increases, the Hgb concentration increases (resulting in increasing eosinophilia), and the cell shape changes from round to ellipsoid. Unlike mammalian RBCs, avian RBCs retain their nucleus.

Avian erythropoietin, a glycoprotein that differs structurally from mammalian erythropoietin, is necessary for the multiplication and differentiation of precursor stem cells committed to the erythroid series.^{18,19} Erythropoietin can be obtained from the blood of anemic birds and the site of its production is considered to be the kidney.

The rubriblast is a large, round, deeply basophilic cell with a large, round central nucleus resulting in a high nuclear to cytoplasmic (N:C) ratio. The nucleus has coarsely granular chromatin and large prominent nucleoli or nucleolar rings. The cytoplasm is deeply basophilic and frequently contains clear spaces that most likely represent mitochondria.

The prorubricyte resembles the rubriblast but lacks prominent nucleoli. It also has a high N:C ratio with the large nucleus being surrounded by a narrow rim of blue cytoplasm. The cytoplasm is predominantly basophilic, but may contain spots of reddish material suggestive of the beginning of hemoglobin development. The cytoplasm lacks the mitochondrial spaces of the rubriblast.

Rubricytes are round cells that are smaller than rubriblasts and prorubricytes. They can be divided into three stages based primarily upon the appearance of the cytoplasm. The basophilic rubricyte is the earliest rubricyte stage, and is characterized by a homogeneous basophilic cytoplasm and a round nucleus with clumped chromatin. The early polychromatophilic rubricyte is smaller than the basophilic rubricyte, and has a gray (basophilic to slightly eosinophilic) cytoplasm because of increased Hgb production. The nucleus of this cell contains clumped chromatin and is small in relation to the amount of cytoplasm. The final rubricyte stage, the late polychromatophilic rubricyte, is ellipsoid with more eosinophilic (eosinophilic gray to weakly eosinophilic) cytoplasm than earlier stages and the nucleus varies from round to slightly ellipsoid with irregularly clumped chromatin.

Cells in the final stages of erythropoiesis are the polychromatophilic RBCs and mature RBCs. These cells are

found in the blood of normal birds and have been previously described.

Granulopoiesis

Avian granulocytes appear to develop in a manner similar to those of mammals; therefore, they show a progressive decrease in size and cytoplasmic basophilia as they mature. Specific cytoplasmic granules appear in later stages of development and progressively increase in number until a full complement is reached in the cytoplasm of the mature granulocyte. The nuclei of granulocytes are initially round and progress toward segmentation, except basophils which do not segment. The nuclear chromatin becomes increasingly condensed with maturity. The developmental stages of avian granulocytes include myeloblasts, progranulocytes, myelocytes, metamyelocytes, band cells, and mature granulocytes, in order of maturation.

The myeloblast is a large round blast cell with a high N:C ratio and its cytoplasm stains a lighter blue than that of rubriblasts. The nucleus is typically round with a delicate reticular (fine) chromatin and prominent nucleoli. Myeloblasts do not contain specific cytoplasmic granules and possibly represent a stage common to all granulocytes. Myeloblasts are frequently found in association with other developing granulocytes, especially in imprints of bone marrow core biopsies.

The progranulocyte is a large cell with a light blue cytoplasm and slightly eccentric nucleus. The N:C ratio of this cell is smaller than that of myeloblasts because of the increase in cytoplasm volume. The nucleus has a delicate reticular chromatin, absence of nucleoli, and indistinct margins. Progranulocytes contain primary granules that vary in appearance among the types of granulocytes. For example, the heterophil progranulocyte contains primary granules that vary in color and shape that often appear as orange or deeply basophilic spheres and rings. In contrast, eosinophil progranulocytes contain only brightly staining orange primary granules and appear to lack the dark magenta granules and rings found in heterophilic progranulocytes. Basophil progranulocytes contain basophilic granules that appear smaller than the specific basophilic granules and the immature granules of the heterophil series. Fewer ring forms are seen in basophil progranulocytes.

The myelocyte is smaller than myeloblasts and progranulocytes. This cell type contains the secondary or specific granules of the mature granulocytes, making identification of this cell somewhat simple. The round to oval nuclei of the myelocyte appears more condensed than the nuclei of myeloblasts and progranulocytes. Heterophil myelocytes are typically round cells with a light blue cytoplasm that contains a mixture of rod-shaped specific granules and primary granules and rings. The eosinophilic, rod-shaped specific granules occupy less than one-half the cytoplasmic volume. Eosinophil myelocytes lack the deeply basophilic granules and rings occasionally found in early heterophil myelocytes. Basophil myelocytes contain basophilic

specific granules that occupy less than one-half the cytoplasmic volume. The specific basophil granules have a slightly eosinophilic tinge compared to the deep violet of the smaller primary granules that may also be present.

The metamyelocyte is slightly smaller than the myelocyte, has a slightly indented nucleus, and possesses specific cytoplasmic granules that occupy greater than one-half the cytoplasmic volume.

Band cells resemble mature granulocytes except that the nucleus appears as a curved or coiled band rather than segmented. It is often difficult to identify band cells because the exact shape of the nucleus is obscured by specific cytoplasmic granules in blood films stained with Romanowsky stains. Because mature basophils lack a segmented nucleus, the band stage of the basophil is not apparent. Mature granulocytes are generally the most abundant cell of each granulocytic cell line in the bone marrow of normal birds and have been described previously.

Thrombopoiesis

Unlike mammalian platelets that are cytoplasmic fragments of large multinucleated megakaryocytes, avian thrombocytes are derived from a distinct line of mononuclear cells found in the bone marrow. The thrombocyte series consists of thromboblats, immature thrombocytes, and mature thrombocytes. Thromboblats resemble rubriblasts but tend to be smaller with round nuclei, fine to punctate nuclear chromatin, and one or more nucleoli. The scant cytoplasm stains deeply basophilic and may contain clear spaces. These cells tend to be round to oval with cytoplasmic blebs.

Immature thrombocytes are divided into three groups: early-, mid-, and late-immature thrombocytes, based upon their degree of maturity. Early-immature thrombocytes are intermediate in size between thromboblats and more mature stages. They tend to be round to oval with more abundant cytoplasm than thromboblats. The cytoplasm is basophilic and may contain vacuoles. The nuclear chromatin is aggregated into irregular clumps. Mid-immature thrombocytes are slightly elongate or irregular with pale blue cytoplasm. Cytoplasmic specific granules and vacuoles are occasionally seen at this stage of development. The nucleus contains heavy chromatin clumping. Late-immature thrombocytes are oval and slightly smaller than the mid-immature stage. The cytoplasm stains pale blue with vaguely defined clear areas. Specific granules are frequently seen at one pole of the cell. The nucleus is oval with densely packed chromatin. The mature thrombocyte is the definitive cell in the thrombocyte series and has been previously described.

Other Cells Present In Avian Bone Marrow

Monocytes and macrophages are frequently found in avian bone marrow samples. Monocytopoiesis is poorly defined in birds. Monocytes originating in hematopoietic tissue become the monocytes and macrophages

found in blood and body tissues, respectively. Because they are involved with iron metabolism during hemoglobin synthesis and catabolism, macrophages in bone marrow frequently contain gray to black (or sometimes golden crystalline) iron pigment in their cytoplasm.

Aggregates of lymphocytes are found in bone marrow of birds, although major sites of lymphopoiesis in adult birds are located in the spleen, liver, intestines, and cecal tonsils.² Immature avian lymphocytes are larger than mature lymphocytes and are classified as lymphoblasts or prolymphocytes, based on morphology. Lymphoblasts have large nuclei with fine chromatin and contain one or more prominent nucleoli. The cytoplasm is relatively abundant and deeply basophilic. Prolymphocytes resemble lymphoblasts but the nuclear chromatin is coarser and nucleoli are absent. Mature lymphocytes have been previously described.

Avian osteoblasts are large cells found in bone marrow and resemble those of mammals. They have abundant, foamy, basophilic cytoplasm with a distinct clear Golgi apparatus. The nucleus is round to oval, eccentrically located in the cell, contains reticular to coarsely granular chromatin, and possesses one or more distinct nucleoli. Osteoblasts are polygonal to fusiform and may have indistinct cytoplasmic margins.

Osteoclasts are large multinucleated giant cells with an amoeboid shape. The cytoplasm is weakly basophilic and vacuolated. Red cytoplasmic granules may be present. Nuclei are round to oval and frequently contain prominent nucleoli.

Cell Distribution

The normal M:E ratio of psittacine birds is presumed to be 1.0:1.0. Studies investigating the normal bone marrow cytology of other species of birds indicate the M:E ratio to be 1.2, with a mean erythroid percentage of 39.9%, a mean myeloid percentage of 49.4%, a mean thrombocyte percentage of 6.0% and a mean percentage of all other cells percentage of 4.8%.²⁰ Increased erythropoiesis is indicated by an increase in polychromasia (reticulocytes) in the blood and bone marrow films. A decrease in the M:E ratio with a normal heterophil concentration in the peripheral blood is an indication of accelerated erythropoiesis. Alternatively, an anemic psittacine bird with a normal peripheral blood heterophil concentration, little or no polychromasia, and an increased M:E ratio is consistent with a decrease in erythropoiesis. Erythroid dysplasia in the peripheral blood film and a marked decrease in the M:E ratio in the bone marrow indicate a myelodysplastic disorder with erythroid predominance. Increased granulopoiesis is usually indicated by a peripheral heterophilia and an

increased bone marrow M:E ratio. A decrease in peripheral blood heterophil concentration and decrease in bone marrow M:E ratio indicate a decreased granulopoiesis. Myeloid leukemias exhibit increased numbers of myeloid blast cells in the peripheral blood and bone marrow.

Starvation can result in a decrease in myeloid, erythroid, and thrombocyte elements, but the bone marrow M:E ratio may be normal. A profound leukopenia, heteropenia, and anemia associated with severe bone marrow suppression and hypoplasia can be associated with toxicities, such as fenbendazole or albendazole toxicity, in birds.²¹

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Hematology of Waterfowl and Raptors

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Waterfowl

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Acronyms and Abbreviations

EDTA, ethylenediaminetetraacetic acid; Hgb, hemoglobin; H:L, heterophil:lymphocyte ratio; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; N:C, nuclear:cytoplasmic ratio; PCV, packed cell volume; RBC, red blood cell; WBC, white blood cell.

WATERFOWL

Erythrocyte Morphology

Mature erythrocytes of waterfowl, like those of other birds, are elliptical cells with an elliptical, centrally positioned purple nucleus that contains uniformly clumped chromatin and a uniform orange-pink cytoplasm in Wright's stained blood films.²¹ In general, mature erythrocytes appear uniform in size in the blood film of birds; however, erythrocyte subpopulations where larger erythrocytes (mean corpuscular volume [MCV] = 308 fL) most likely represent those most recently released from the hematopoietic tissue and smaller cells (MCV = 128 fL) likely representing older aging cells have been reported in ducks.¹³

Polychromatophilic erythrocytes represent cells recently released into the peripheral blood. As these cells mature, the nuclear chromatin becomes more condensed and the cytoplasm less blue. Polychromatic erythrocytes are more prevalent in the peripheral blood of waterfowl compared to mammals because the erythrocyte lifespan of birds is generally shorter in comparison to that of mammalian erythrocytes. For example, the erythrocyte lifespan of ducks is 42 days.³⁵

Erythrocytes that vary in size from the majority of erythrocytes are occasionally observed in peripheral blood films of normal waterfowl; however, a greater degree of anisocytosis as well as polychromasia is observed in birds with a regenerative response to anemia.

Likewise, a minor deviation from the normal shape of erythrocytes is considered normal in the peripheral blood of birds; however, marked poikilocytosis is an indication of erythrocytic dysgenesis.

Hypochromatic erythrocytes are occasionally found in the peripheral blood film of normal waterfowl; however, when they occur in high numbers, it is considered abnormal and a significant hypochromasia will be reflected as a decrease in the mean corpuscular hemoglobin concentration (MCHC).

Hypochromasia can be seen with iron deficiency, chronic inflammatory diseases and lead toxicosis.^{11,20} Hypochromatic erythrocytes frequently appear in blood films from waterfowl with chronic inflammatory diseases, presumably related to iron sequestration as part of the bird's defense against infectious agents. In such cases, hypochromatic cells are often observed in blood films before the red cell indices, such as MCHC, suggest hypochromasia.

The presence of immature erythrocytes (i.e. rubricytes) in peripheral blood films of waterfowl, in addition to increased polychromasia, indicates a marked erythrocytic regenerative response as seen with hemolytic or blood loss anemias.⁴⁰

A nonregenerative normocytic normochromic anemia is indicative of decreased erythropoiesis (depression anemia) which can develop rapidly in birds with inflammatory diseases, especially those involving infectious agents, owing to their short erythrocyte half-life. Thus, the degree of polychromasia or reticulocytosis is poor to absent in birds with depression anemias.

Leukocyte Morphology

Leukocytes in blood films of waterfowl, like those of other birds, include lymphocytes, monocytes, and granulocytes. The granulocytes are classified as heterophils, eosinophils, and basophils. In general, the morphology of these cells is the same as that of other birds (Chapter 123).²¹

In the species of Anseriformes (ducks and geese) whose normal leukograms have been studied, lymphocytes have been shown to be the most abundant leukocyte.^{7,40} For example, the heterophil:lymphocyte ratio (H:L) for the American black duck, wood duck, mallard, and snow goose ranges between 0.4:1 and 0.7:1.^{7,22,23,39}

Lymphocytes of waterfowl, like those of other birds, can be small or large. Reactive lymphocytes are occasionally found in the blood films of normal birds. These lymphocytes have a deep staining blue cytoplasm, often with an irregularly shaped nucleus and pink-purple cytoplasmic granules, compared to nonreactive lymphocytes. Reactive lymphocytes without the cytoplasmic granules are considered to be B cells, whereas those with granules are thought to be natural killer or T cells.

Thrombocyte Morphology

Like those of all birds, the blood films of waterfowl contain thrombocytes. The morphology of these cells is consistent among the various species of birds. Because thrombocytes participate in the coagulation process, they tend to clump in the blood film.

Reference Intervals

These are given in Table 124.1.

Age Influences

Age can be a cause of variation in the normal hemogram of healthy waterfowl. For example, 4- to 10-week-old ducklings exhibit a lower packed cell volume (PCV) and slightly higher protein concentration compared to adult ducks.¹⁸ This difference is likely associated with the high metabolic rate of young rapidly growing ducks that are consuming diets higher in protein and carbohydrates.

Age may also affect the normal leukogram. For example, ducklings and goslings younger than 60 days of age have lower relative lymphocyte and higher relative heterophil counts compared to adult ducks and geese.⁷

Age also influences the thrombocyte count. Young ducklings and goslings tend to have lower thrombocyte concentrations compared to adult birds of the same species. The number of thrombocytes in the peripheral blood begins to increase at 5 days of age and reaches adult concentrations by 18 days of age.⁷

Species Influences

Significant differences occur between species of waterfowl in regard to the red blood cell (RBC) parameters. For example, mallard ducks (*Anas platyrhynchos*), a dabbling duck, have higher average PCV and RBC counts in the winter and pre-nesting period compared to diving ducks (*Aythya* spp. and *Oxyura jamaicensis*).³¹ The same study demonstrated that diving ducks, on the other hand, have higher MCV values during the winter and pre-nesting period compared to mallards. In general, ducks tend to have higher RBC counts than geese, but geese have higher MCV and hemoglobin (Hgb) than ducks during the winter.³¹ Total RBC counts tend to be slightly higher for snow geese (*Chen caerulescens*) compared to Canada geese (*Branta canadensis interior*); however, these differences are clinically insignificant.³⁹

Birds with a normal H:L ratio less than 1.0, such as many species of waterfowl, exhibit an initial leukogram response to stress and inflammation with leukopenia associated with a lymphopenia followed up to 12 hours later by a leukocytosis, heterophilia, and lymphopenia.^{5,10} Birds with a higher proportion of heterophils than lymphocytes in their normal leukograms tend to exhibit a less dramatic response in the stress leukogram.

Immature heterophils are rarely found in the blood film of normal waterfowl. Immature heterophils have increased cytoplasmic basophilia, nonsegmented nuclei, and immature cytoplasmic granules when compared to normal mature heterophils (see Chapter 123). When present, they represent a left shift indicating an increased demand for heterophils owing to an inflammatory stimulus and can be associated with either a heterophilia or heteropenia. Leukemia would be a rare cause for the presence of immature heterophils in the blood film of waterfowl.

In response to severe systemic inflammation, avian heterophils exhibit toxic changes similar to those seen in mammalian neutrophils.³ Also, as in mammalian hematology, toxic changes in avian heterophils are subjectively quantified as to the number of toxic cells and severity of toxicity.³⁸ When present, toxic heterophils represent a severe inflammatory response regardless of the leukocyte count.

Physiological and Environmental Influences

Like those of other avian species, the normal values for the hematologic parameters of waterfowl have a broad

TABLE 124.1 Selected Hematology Data for Normal Ducks and Geese

	American Black Duck ^a	Wood Duck ^b	Canada Goose ^c		Snow Goose ^d	Mallard Duck ^e	
			January	June		January	June
RBC ($\times 10^6/\mu\text{L}$)	2.78 \pm 0.22	2.79 \pm 0.22	2.67 \pm 0.3	2.18 \pm 0.3	2.25	3.35 \pm 0.3	2.01 \pm 0.4
PCV (%)	40.24 \pm 4.21	45.54 \pm 3.41	51 \pm 3.1	46 \pm 3.5	46	49 \pm 2.5	39 \pm 5.1
Hb (g/dL)	12.96 \pm 1.36	14.95 \pm 1.22	16.2 \pm 1.0	14.0 \pm 1.2	14.0	15.6 \pm 0.8	11.4 \pm 1.6
MCV (fL)	144.68 \pm 9.96	164.24 \pm 14.43	193.3 \pm 16.6	210.1 \pm 19.4	204	148.4 \pm 13.7	199.6 \pm 27.7
MCHC (g/dL)	32.23 \pm 1.16	32.99 \pm 3.7	31.6 \pm 0.1	30.7 \pm 1.4	30.4	31.6 \pm 0.2	29.2 \pm 2.0
WBC ($\times 10^3/\mu\text{L}$)	19.7 \pm 6.60	25.58 \pm 5.72	20.4–21.8		20.1 \pm 4.71	23.4–24.8	
Heterophils ($\times 10^3/\mu\text{L}$)	4.86 \pm 1.37	8.45 \pm 2.59	39%		7.0	38% \pm 1.5	29% \pm 1.4
Lymphocytes ($\times 10^3/\mu\text{L}$)	13.03 \pm 1.53	13.28 \pm 1.77	46%		12.3	54% \pm 1.6	66% \pm 1.4
Monocytes ($\times 10^3/\mu\text{L}$)	1.46 \pm 0.99	1.05 \pm 0.68	6.0%		0.2	3.6% \pm 0.3	2.5% \pm 0.3
Eosinophils ($\times 10^3/\mu\text{L}$)	0.22 \pm 0.16	0.51 \pm 0.06	2.0%		0.5	0.4% \pm 0.1	0.2% \pm 0.17
Basophils ($\times 10^3/\mu\text{L}$)	0.16 \pm 0.15	0.41 \pm 0.23	7.0%		0.1	3.6% \pm 0.3	2.2% \pm 0.3

^aMean \pm SD. Reference 22.^bMean \pm SD. Reference 23.^cMean \pm SEM. References 31, 37.^dMean values. Reference 39.^eMean \pm SEM. References 7, 31, 37.

range owing to the physiological and environmental influences on the blood cells (Chapter 123). Seasonal variations, age, and species affect the erythrocyte parameters, such as the PCV, RBC count, Hgb concentration, MCHC, and MCV of normal waterfowl. In general, variations in these parameters in adult ducks and geese most likely reflect seasonal effects associated with hormonal and nutritional changes caused by alterations in the photoperiod and migration. For example, variations in erythrocyte parameters associated with gender in ducks and geese are generally not statistically significant; however, differences between males and females have been reported and reflect a seasonal variation, where females tend to have higher PCV, Hgb, RBC counts, and MCHC values compared to males in the pre-nesting period.^{22,23,38} Other studies have shown that averages for these parameters tend to be higher in the winter and pre-nesting period in adult ducks and geese regardless of gender compared to the post-nesting period and fall.^{31,39} During migration, ducks tend to have slightly lower RBC counts compared to wintering ducks and post-nesting MCV averages for these birds tend to be higher in the winter or pre-nesting periods.^{18,31} These changes also occur in captive ducks that are not able to migrate.

In general, the leukogram (white blood cell [WBC] count and H:L ratio) is not affected significantly by the reproductive state (pre-egg laying, egg-laying, incubation, molting, and post-egg laying) of ducks and geese, and there is no significant difference between genders.^{7,39} However, the percentage of heterophils in the periph-

eral blood of ducks has been shown to decrease during and immediately following the molting of the flight feathers (remige molt).⁶

Thrombocyte concentrations are also influenced by seasonal changes. For example, a decrease in thrombocyte concentration has been demonstrated in female ducks during the incubation, molting, and post-reproductive periods compared to other periods.⁷

Blood Sampling Conditions

Blood collection for hematologic evaluation in waterfowl requires proper restraint. In general, these birds are relatively harmless to humans, with large geese and swans being an exception. Most can be physically restrained safely in a cloth towel without anesthesia before venipuncture as long as excessive restraint around the keel is avoided to prevent asphyxiation. Excessive stress that occurs with the capture and restraint of birds, especially those not accustomed to being handled or birds that are ill, can further compromise the health of the patient and the hematologic indices.

A maximum blood sample representing 1% of the bird's body mass (or 10% of the blood volume) is generally considered to be a safe amount of blood that can be obtained from birds. For routine hematological evaluations in birds, a sample size of 0.2 mL is usually adequate.⁴

A variety of venipuncture sites for blood collection in waterfowl are available and include the jugular,

basilic vein (cutaneous ulnar, wing or brachial), and the medial metatarsal (caudal tibial) vein. Blood can be collected using the same procedure described for other avian species (Chapter 123).

Bone Marrow Sampling

Collection Sites

Marrow samples for cytological evaluation can be successfully obtained in waterfowl by bone marrow aspiration of the proximal tibiotarsus or other non-pneumatic long bones using the same technique as for other birds (Chapter 123).

Cell Morphology

The study of avian hematopoiesis lags behind research in mammalian hematopoiesis because the maturation stages of avian granulocytes have been described based only upon their morphologic appearance, primarily in chicken bone marrow.

Erythropoiesis The stages of maturation in normal avian erythropoiesis appear to be similar to those of mammals. The terminology used for the different stages of erythrocyte maturation varies in the literature.^{3,8,11} In general, there are six recognizable stages involved in red blood cell development based upon Romanowsky stains. These include rubriblasts, prorubricytes, basophilic rubricytes, early polychromatic rubricytes, late polychromatic rubricytes, polychromatic erythrocytes, and mature erythrocytes (Figs.124.1–124.3). As erythroid cells mature, the nuclear size decreases, the chromatin becomes increasingly condensed, the nuclear shape changes from round to ellipsoid, the amount of cytoplasm increases, the Hgb concentration increases (resulting in increasing eosinophilia), and the cell shape changes from round to ellipsoid. Unlike mammalian erythrocytes, avian erythrocytes normally retain their nucleus.

Avian erythropoietin, a glycoprotein that differs structurally from mammalian erythropoietin, is necessary for the multiplication and differentiation of precursor stem cells committed to the erythroid series.³⁵ Erythropoietin can be obtained from the blood of anemic birds and the site of its production is considered to be the kidney.

Granulopoiesis The developmental stages of avian granulocytes include myeloblasts (granuloblasts), progranulocytes (promyelocytes), myelocytes, metamyelocytes, band cells, and mature granulocytes, in order of maturation. (Fig. 124.4) In general, avian granulocytes show a progressive decrease in size and cytoplasmic basophilia as they mature, similar to granulocytes of mammals. Specific cytoplasmic granules appear during the later stages of development and progressively increase in number until a full complement is reached in the cytoplasm of the mature granulocyte. The nuclei of avian granulocytes are initially rounded, and progress

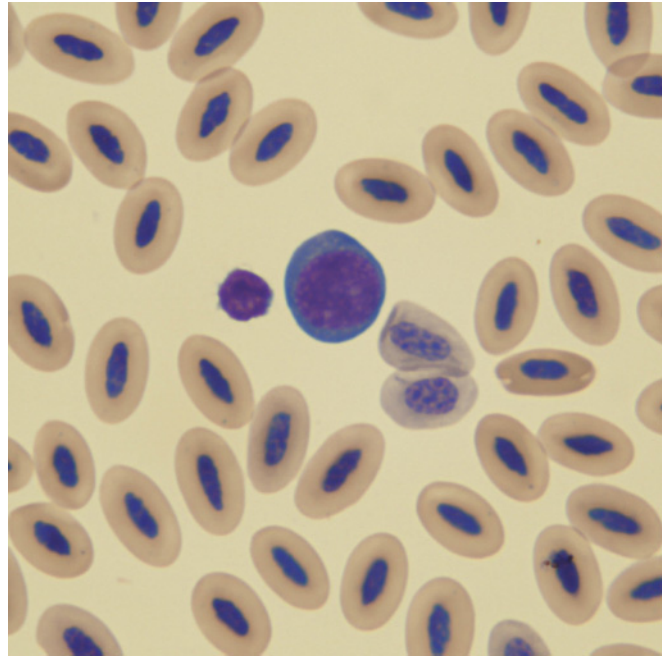


FIGURE 124.1 A prorubricyte in the bone marrow sample of a duck. Wright stain.

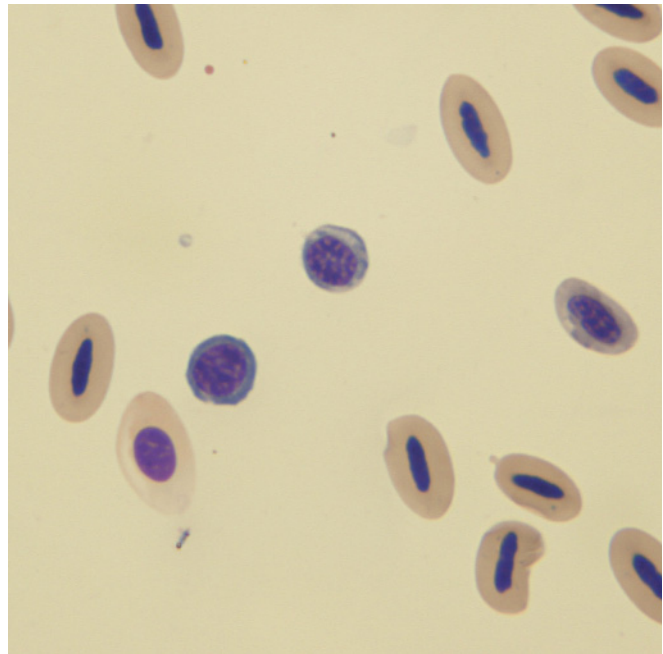


FIGURE 124.2 A basophilic (bottom round cell) and an early polychromatic rubricyte (top round cell) in the bone marrow sample of a duck. Wright stain.

toward segmentation and the nuclear chromatin becomes increasingly condensed with maturity. Basophils are the exception because the nucleus of the avian basophil does not segment with maturation.

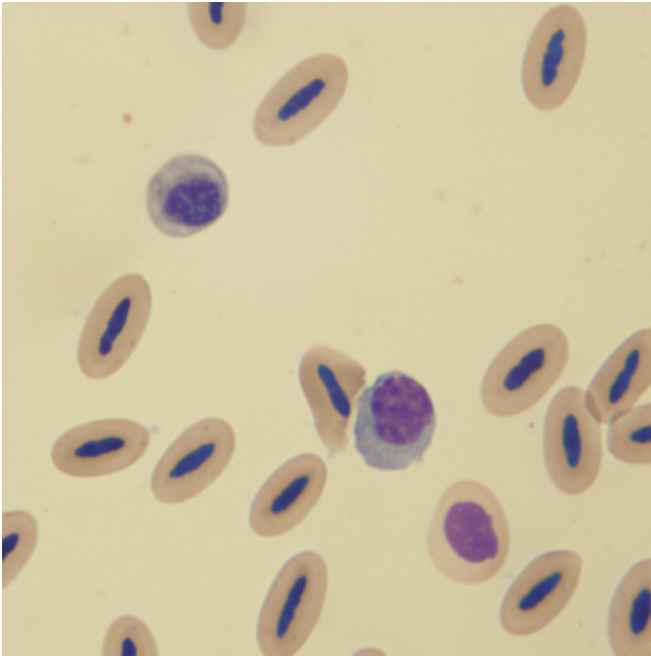


FIGURE 124.3 A late polychromatic rubricyte (top round cell) in the bone marrow sample of a duck. Wright stain.

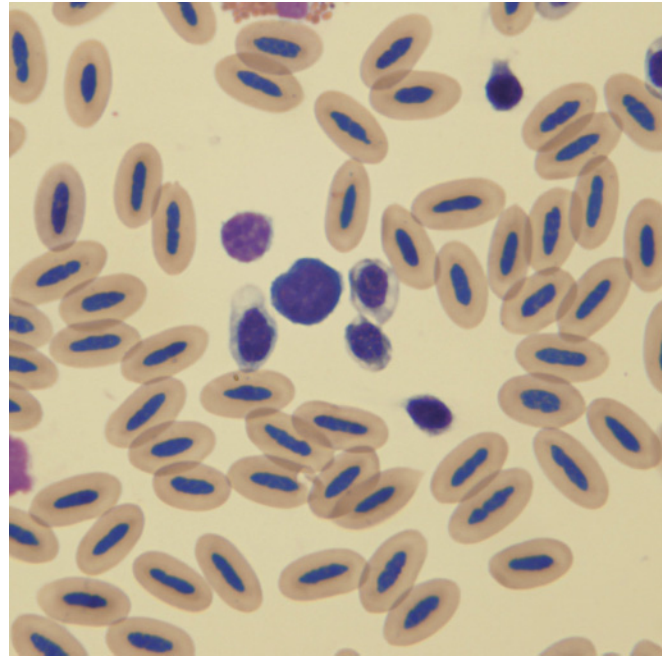


FIGURE 124.5 A thrombobl原因 (dark cell in the center) flanked by mature thrombocytes in the bone marrow sample of a duck. Wright stain.

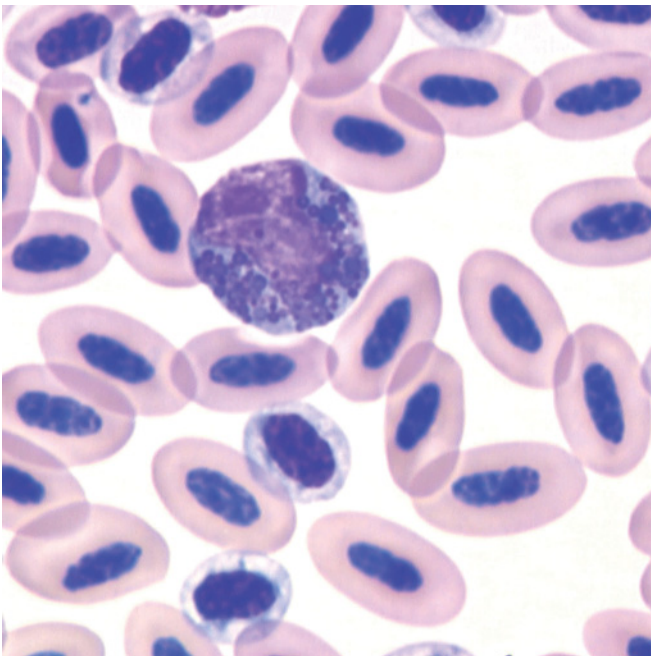


FIGURE 124.4 A progranulocyte in the bone marrow sample of a duck. Wright stain.

Thrombopoiesis Avian thrombocytes appear to be derived from a distinct line of mononuclear cells found in the bone marrow, unlike mammalian platelets which are cytoplasmic fragments of large, multinucleated megakaryocytes. The avian thrombocyte series consists of thrombobl原因, immature thrombocytes, and mature thrombocytes (Fig. 124.5). Thrombobl原因 appear as

small, round to oval cells that resemble rubriblasts, and contain round nuclei with fine to punctate nuclear chromatin, and one or more nucleoli. Cytoplasmic blebs may be present on the cell margins.

Immature thrombocytes may be divided into three groups: early-, mid-, and late-immature thrombocytes based upon their degree of maturity. Avian thrombocytes show a progressive decrease in cell size, nuclear size, and cytoplasmic basophilia as they mature. There is also a progression toward the ovoid shape of the mature cell and the nucleus becomes more pyknotic with age. Cytoplasmic specific granules and vacuoles appear at the mid-immature thrombocyte stage of development.

RAPTOR HEMATOLOGY

Raptor, or birds of prey, is an informal grouping of several families including: Accipitridae (hawks, eagles, buzzards, harriers, kites, Old World vultures), Pandionidae (osprey), Sageittaridae (secretary bird), Falconidae (falcons, caracaras), Cathartidae (New World vultures, condors), Strigidae (typical owls), and Tytonidae (barn and bay owls).² Literature regarding hematological data from raptorial birds is limited. Additionally, most studies do not provide enough information for the establishment of proper reference intervals, usually due to a small sample size and lack of medically pertinent information, such as age, sex, health, season, and breeding status. Therefore, some clinicians suggest that only significantly abnormal blood values which correspond with clinical findings in support of a disorder be considered valid.¹² However,

the lack of established hematology reference intervals on birds of prey should not deter the clinician from using hematology as a diagnostic tool.

As with other avian species, the blood of raptors contains nucleated erythrocytes, nucleated thrombocytes, and various types of leukocytes. The normal avian hemogram usually includes PCV, RBC and WBC counts, and the determination of the Hgb concentration. Factors that may influence the hematologic results include individual variation between species, wild versus captive status, age, sex, nutrition, environmental conditions and health status.¹²

Blood Sampling Condition

Common sites of collecting blood from raptors include the cutaneous ulnar (wing) vein, jugular vein, or medial metatarsal vein. Even with large birds of prey such as eagles, hawks and owls, only short 25–22 gauge needles should be used to obtain blood samples as the vessel walls of birds are relatively thin and easily damaged.⁴ Larger volumes of blood can be collected via cardiac puncture or occipital venous sinus but with risk of serious complications. Adequate restraint of the bird is essential to prevent the inadvertent formation of hematomas from lacerated vessels. Blood should be immediately used to make fresh smears or drawn into tubes containing EDTA anticoagulant, as heparin often causes clumping of leukocytes and changes in the affinity of cells to Romanowsky (Wright's or Wright-Giemsa) stains.¹⁶ Ethylenediaminetetraacetic acid (ETDA) has been associated with mild hemolysis in some species, in which cases heparinized tubes can be used with the added advantage that the plasma can be used for blood chemistry analysis.⁴

Laboratory Evaluation

The PCV can be determined by use of a microhematocrit capillary tube and centrifugation as with mammalian blood. Total RBC counts can be determined manually by using a hemocytometer and erythrocyte Unopette (Becton-Dickinson, Rutherford, NJ) method. Directions for use of other special diluents (Natt-Herrick's) for counting avian blood cells can be found in Chapters 50 and 123 of this book. Properly calibrated, automated cell counters can also be used with a higher degree of precision for rapidly determining RBC counts in avian species.¹⁶ The WBC count can be performed manually using either Natt-Herrick's solution or the eosinophil Unopette 5877 (Becton-Dickinson) method. The free nuclei from lysed RBCs interfere with the cyanomethemoglobin spectrophotometric measurement of Hgb in avian blood samples. Therefore, as with other avian species, the blood sample must be centrifuged following cell lysis to remove the free nuclei before evaluating Hgb concentration. Following determination of total RBC number, PCV, and Hgb concentration, the MCV, MCH, and MCHC are calculated as for other species.⁴

Erythrocyte Morphology

The raptorial RBC, like those of other avian species, is an elliptical, biconvex cell that contains an elongated oval nucleus. The centrally located nucleus has clumped chromatin which becomes more condensed as the cell ages.¹⁶ Immature polychromatophilic erythrocytes have a clear light blue cytoplasm and may constitute up to 5% of the cell count in mature birds with normal RBC mass with higher percentages seen in association with increased erythropoiesis in responsive anemia (Fig. 124.6). With vital stain (new methylene blue) polychromatophilic red cells will stain as reticulocytes with prominent dark aggregates of reticulin material surrounding the nucleus. Reticulocytes mature rapidly to normochromic erythrocytes which have a relatively short lifespan of 20–45 days.¹⁵

The PCV of raptors ranges from 30% to 50%, with an average being around 40% (Table 124.2). Redig reported hematocrits of wild raptors as being between 42% and 45%, with values of 50% common in the larger falcons.²⁸ The MCV in raptors and most avian species is considerably higher than in mammals, ranging from 105–220 fL.^{16,34} Smith and Bush found that the RBCs for hawks and falcons were slightly smaller and more numerous than those of eagles and owls.³² Generally, the RBC counts in raptors (species range $1.5\text{--}5.4 \times 10^6/\mu\text{L}$) are also lower than seen in mammalian blood, while Hgb concentration is similar.^{4,14,16} Variation in MCHC and mean corpuscular hemoglobin (MCH) from mammalian values is secondary to differences in raptor RBC counts and MCV.

Unlike pigeons and doves, no apparent difference existed in the PVC and RBC values between the sexes of the same species of raptor.³³ This same study also demonstrated no significant differences in these values for kestrels maintained under different lighting regimens or of differing breeding experience (i.e. juvenile versus breeding).

Leukocyte Morphology

The leukocytes consist of granulocytes (heterophils, eosinophils, basophils), lymphocytes, and monocytes (Figs. 124.7, 124.8). Heterophils are round cells 10–12 μm in diameter with a lobulated nucleus. The cytoplasm is usually colorless to pale pink and contains numerous rod-shaped to oval eosinophilic granules which may be partially refractile. Lymphocytes vary in size and shape but are generally approximately the size of erythrocytes or slightly smaller, and have a large, dark-staining nucleus with scant basophilic cytoplasm that lacks vacuoles and granules. The eosinophil is an irregularly rounded cell similar in size to the heterophil with a clear to light blue cytoplasm and a bilobed nucleus. These cells contain intensely eosinophilic spherical inclusions that are generally more numerous than those of the heterophil. Basophils are similar to heterophils in size and shape but contain strongly basophilic granules. Monocytes are slightly larger than lymphocytes with a large, less darkly staining, variably shaped nucleus and

TABLE 124.2 Referenced Raptor Erythrocyte Parameters

Species	RBC ($\times 10^6/\mu\text{L}$)		PCV (%)		Hgb (g/dL)		MCV (fL)		MCH (pg)		MCHC (g/dL)		Reference
	Mean \pm S.D.	Range	Mean \pm S.D.	Range	Mean \pm S.D.	Range	Mean \pm S.D.	Range	Mean \pm S.D.	Range	Mean \pm S.D.	Range	
Owls													
Tawny	2.04	1.50–2.42	38	29–47	10.3	0.8–13.3	189	154–221	47.3	33.1–62.1	25.6	19.3–31.4	34
Northern eagle	—	1.65–2.35	—	36–52	—	10.7–18	—	189–204	—	64.6–76	—	32.5–37.6	16
Eagles													
Bald	—	—	32	25–41	11.83	9.10–16.30	—	—	—	—	37.4	27.70–53.70	1
Spanish imperial	2.19	1.93–2.45	43	39–46	14.8	12.6–16.9	200	181–214	68	58–78	34	28–37	9
Golden	1.63 \pm 0.11	—	47 \pm 0.9	—	9.2 \pm 0.1	—	—	—	—	—	—	—	25
Spanish imperial	2.41 \pm 0.15	2.20–2.74	43.7 \pm 3.6	37.0–49.5	13.6 \pm 1.4	13.5–16.2	179.5 \pm 10.5	157.0–190.7	56.7 \pm 4.2	49.0–63.2	31.3 \pm 1.4	29.3–33.3	26
Golden	2.56 \pm 0.54	1.96–3.22	42.1 \pm 3.7	38.0–46.0	13.8 \pm 1.3	12.5–15.2	168.2 \pm 18.6	150.9–187.8	44.0 \pm 12.7	47.1–63.7	32.8 \pm 0.3	32.7–33.1	26
Golden	—	1.69–3.21	—	31–52	—	11.2–17.3	—	165–186	—	53.8–67.7	—	32.6–36.4	16
Tawny	—	2.32–2.83	—	37–47	—	10.8–17.5	—	163–188	—	54–62	—	296–360	16
Hawks/Falcons													
Common buzzard	2.94 \pm 0.82	2.06–5.44	40.8 \pm 4.4	36–49	12.7 \pm 2.4	9.3–17.7	145.1 \pm 25.0	90.0–171.4	48.3 \pm 10.2	32.5–65.7	32.4 \pm 6.7	22.6–45.3	14
Sharp-shinned	—	—	47.6 \pm 6.73	30–76	—	—	—	—	—	—	—	—	27
Peregrine falcon	2.17 \pm 0.07	1.27–2.86	37.58 \pm 0.82	31.8–44.84	20.96 \pm 0.29	17.54–23.0	179.37 \pm 8.24	123.90–304.83	100.16 \pm 4.43	72.01–160.56	56.56 \pm 1.54	42.53–68.69	19
Gyr falcon (wild)	3.91 \pm 0.14	3.2–5.12	51.36 \pm 0.90	44–59	18.85 \pm 0.23	16.0–21.2	135.83 \pm 3.59	106.18–162.36	49.44 \pm 1.32	39.17–59.67	36.41 \pm 0.16	35.47–37.84	29
Gyr falcon (captive)	3.23 \pm 0.28	—	45 \pm 0.04	—	15.0 \pm 1.33	—	139.32 \pm 5.44	—	45.78 \pm 1.84	—	—	—	29
Lanner falcon	—	2.63–3.98	—	37–53	—	12.2–17.1	—	127–150	—	42.3–48.8	—	31.7–35.3	16
Lagger falcon	—	2.65–3.63	—	39–51	—	12.8–16.3	—	123–145	—	38–47.7	—	31.2–35.0	16
Merlin falcon	—	2.85–4.1	—	39–51	—	13.2–17.9	—	105–130	—	36–45.9	—	34.0–36.0	16
Peregrine falcon	—	2.95–3.94	—	37–53	—	11.8–18.8	—	118–146	—	40–48.4	—	31.9–35.2	16
Saker falcon	—	2.54–3.96	—	38–49	—	11.5–16.5	—	124–147	—	41.4–45.4	—	30.4–34.9	16
Ferruginous hawk	—	2.41–3.59	—	37–48	—	10.7–16.6	—	150–178	—	46–57.4	—	29.7–34.5	16
Redtailed hawk	—	2.2–3.35	—	35–53	—	12.3–17.5	—	157–168	—	43–50.4	—	31.2–35.0	16
Harris' hawk	—	2.63–3.5	—	40–55	—	12.1–17.1	—	147–163	—	45.4–51.1	—	30.1–33.0	16
Eurasian buzzard	—	2.13–2.76	—	32–44	—	10.1–16.7	—	151–165	—	48–53	—	30.7–33.9	16
Northern goshawk	—	2.6–3.48	—	43–53	—	12.1–17.7	—	141–156	—	44.5–51.6	—	30.5–34.3	16
Vultures													
African whitebacked	1.39 \pm 0.44	0.77–2.76	41.78 \pm 4.10	35.35–49.85	13.78 \pm 0.64	12.90–15.20	161.30 \pm 14.85	113.5–188.5	56.76 \pm 8.35	45.15–74.36	33.65 \pm 7.08	20.09–45.45	36
African whitebacked	2.55 \pm 0.22	2.01–3.03	44.30 \pm 4.79	31.0–53.0	19.66 \pm 1.68	14.20–24.10	196.70 \pm 5.55	182.0–211.0	—	—	39.30 \pm 1.53	35.4–42.0	24
Griﬃon	2.63 \pm 0.32	2.19–2.87	45.5 \pm 3.4	40.0–51.8	15.1 \pm 1.9	12.4–18.2	170.0 \pm 15.5	150.9–189.3	55.3 \pm 6.7	46.8–66.6	32.3 \pm 2.5	29.7–36.6	26
Egyptian	2.30 \pm 0.44	1.94–2.94	42.5 \pm 3.6	38.0–48.0	13.7 \pm 1.2	12.4–15.5	187.7 \pm 21.7	163.3–212.6	60.2 \pm 6.3	52.6–67.3	32.2 \pm 0.4	31.6–32.6	26

TABLE 124.3 Referenced Raptor Leukocyte Parameters

Species	WBC		Heterophils			Lymphocytes			Monocytes			Eosinophils			Basophils		
	($\times 10^3/\mu\text{L}$)	($\times 10^3/\mu\text{L}$)	(%)	(%)	($\times 10^3/\mu\text{L}$)	(%)	(%)	($\times 10^3/\mu\text{L}$)	(%)	(%)	($\times 10^3/\mu\text{L}$)	(%)	(%)	($\times 10^3/\mu\text{L}$)	(%)	(%)	($\times 10^3/\mu\text{L}$)
	Mean	Range	Mean	Range	Range	Mean	Range	Range	Mean	Range	Range	Mean	Range	Range	Mean	Range	Range
Owls																	
Tawny ³⁴	10	6.7–15.5	4.28	1.55–9.64	—	4.06	2.05–7.18	—	0.18	0.0–0.47	—	1.27	0.15–3.04	—	0.2	0.07–0.44	—
Barn ¹⁷	—	12.0–20.0	—	15–50	—	—	40–70	—	—	0–3.0	—	—	5.0–25.0	—	—	0–2.0	—
Great Horned ¹⁷	—	10.0–22.0	—	20–60	—	—	20–60	—	—	0–3.0	—	—	5.0–30.0	—	—	0–2.0	—
Eagles																	
Northern eagle ¹⁶	—	3.5–12.1	—	—	2.2–9.23	—	—	1.5–5.07	—	—	0–0.48	—	—	0–0.48	—	—	0–0.35
Bald ¹	17.21	4.62–32.47	44	19–61	—	38	23–60	—	4	0–8	—	13	4.0–26.0	—	—	—	—
Spanish imperial ⁹	15.4	11.7–19.2	51.5	41.0–66.0	—	35.5	26.0–48.0	—	5	1.0–8.0	—	8.5	3.0–16.0	—	0	0	—
Golden ²⁵	24.31 ± 1.97	—	4.40 ± 0.22	—	—	16.8 ± 0.65	—	—	0.99 ± 1.19	—	—	2.10 ± 0.30	—	—	—	—	—
Spanish imperial ²⁶	14.87 ± 6.30	3.38–25.13	60.57 ± 13.10	37.96–82.61	—	30.79 ± 11.27	13.04–52.78	—	1.28 ± 0.71	0.0–2.65	—	6.94 ± 2.92	3.48–11.29	—	0	0	—
Golden ¹⁶	12.32 ± 7.95	5.87–24.0	58.83 ± 9.75	49.0–68.5	—	34.17 ± 5.80	27.5–38.0	—	4.34 ± 4.16	0.0–9.0	—	2.33 ± 1.15	1.0–3.0	—	0.20 ± 0.45	0.0–1.0	—
Tawny ¹⁶	—	6.2–17	—	—	4.5–15.2	—	—	0.75–3.37	—	—	0–0.63	—	—	0.1–0.6	—	—	0–0.16
	—	5–9.5	—	—	3.58–6.45	—	—	0.51–2.72	—	—	0.2–1.07	—	—	0.3–2.1	—	—	0–0.4
Hawks/Falcons																	
Common buzzard ¹⁴	8.04 ± 1.77	4.6–10.6	63 ± 13.1	45–75	—	20 ± 9.5	10.0–48.0	—	0 ± 1.0	0–4.0	—	16.0 ± 13.8	0–37	—	0 ± 0.7	0.0–3.0	—
Sharp-shinned hawk ²⁷	—	—	27.0 ± 14.2	5.0–69	—	63.4 ± 14.4	28–90	—	1.60 ± 1.99	0.0–10.0	—	7.67 ± 4.18	0.0–18.0	—	0.40 ± 0.79	0.0–4.0	—
Common buzzard ²⁷	—	14.0–49.0	—	20.5–39.8	—	—	35–65.5	—	—	0.25–3.75	—	—	5.5–19	—	—	0.25–8.0	—
Marsh harrier ²⁷	—	9.0–33.0	—	26.5–39.5	—	—	48–59.5	—	—	2.5–10.5	—	—	1.5–6.5	—	—	2.8–5.3	—
Common kestrel ²⁷	—	14.5–57.0	—	11.3–33	—	—	24–57.5	—	—	0.25–3.0	—	—	8.75–59.3	—	—	1.5–3.8	—
Black kite ²⁷	—	10.0–28.0	—	28.8–35.3	—	—	29.5–50.5	—	—	0.0–2.0	—	—	12.8–35.5	—	—	2.3–3.5	—
Peregrine falcon ¹⁹	21.26 ± 1.30	11.55–39.23	61.14 ± 2.50	38.80–82.65	—	34.89 ± 2.54	12.90–58.65	—	1.53 ± 0.26	0.0–4.55	—	2.18 ± 0.31	0.0–5.55	—	0.07 ± 0.04	0.0–1.0	—
Gyr falcon (wild) ²⁹	7.3 ± 0.38	4.2–10.8	—	—	2.31–8.85	—	—	0.48–2.36	—	—	0.03–0.9	—	—	0–0.68	—	—	0–0.29
Gyr falcon (captive) ²⁹	8.71 ± 0.38	—	58.53 ± 12.98	—	—	37.54 ± 12.98	—	—	3.72 ± 2.5	—	—	0.2	—	—	0	—	—
Lanner falcon ¹⁶	—	3.5–11	—	—	1.65–8.8	—	—	1.1–5.13	—	—	0–0.9	—	—	0–0.2	—	—	0–0.45
Laggar falcon ¹⁶	—	5–9	—	—	3.5–6.57	—	—	1.7–4	—	—	0–0.85	—	—	0–0.2	—	—	0.17–0.83
Merlin falcon ¹⁶	—	4–9.5	—	—	3.2–4.03	—	—	1.2–1.56	—	—	0–0.5	—	—	0–0.15	—	—	0–0.15
Peregrine falcon ¹⁶	—	3.3–11	—	—	1.4–8.55	—	—	1.1–3.3	—	—	0.1–0.86	—	—	0–0.3	—	—	0–0.6
Saker falcon ¹⁶	—	3.8–11.5	—	—	2.6–5.85	—	—	0.8–4.25	—	—	0–0.8	—	—	0–0.2	—	—	0–0.45
Ferruginous hawk ¹⁶	—	4.5–6.8	—	—	1.89–3.76	—	—	0.78–1.74	—	—	0.24–1.5	—	—	0.3–0.7	—	—	0.15–0.6
Red tailed hawk ¹⁶	—	3.4–7.5	—	—	1.9–3.5	—	—	1.3–1.1	—	—	0.12–1.2	—	—	0.1–0.9	—	—	0–0.5
Harris' hawk ¹⁶	—	4.8–10	—	—	2.3–6.71	—	—	0.62–2.36	—	—	0.2–1.49	—	—	0–0.75	—	—	0–1.55
Eurasian buzzard ¹⁶	—	5–13	—	—	3.2–11	—	—	0.3–3.1	—	—	0.2–0.68	—	—	0.1–0.8	—	—	0–0.9
Northern goshawk ¹⁶	—	4–11	—	—	3.5–6.97	—	—	1.38–1.93	—	—	0–0.1	—	—	0–0.65	—	—	0–0.35
Vultures																	
African whitebacked ²⁴	16.71 ± 1.63	4.0–34.0	13.70 ± 6.11	3.04–27.60	—	1.02 ± 1.91	0–12.55	—	1.57 ± 1.04	0.10–5.78	—	0.75 ± 0.71	0–3.68	—	0	0–0	—
Griﬃon ²⁶	13.19 ± 7.32	5.0–24.0	57.83 ± 9.87	51.0–72.0	—	40.67 ± 10.52	24.0–49.0	—	0.57 ± 0.53	0.0–1.0	—	0.86 ± 1.57	0.0–4.0	—	0	0–0	—
Egyptian ²⁶	16.03 ± 4.19	10.63–21.88	69.75 ± 13.50	58.0–64.0	—	27.25 ± 12.70	9.0–38.0	—	0.50 ± 0.58	0.0–1.0	—	2.0 ± 0.82	1.0–3.0	—	0.40 ± 0.54	0.0–1.00	—

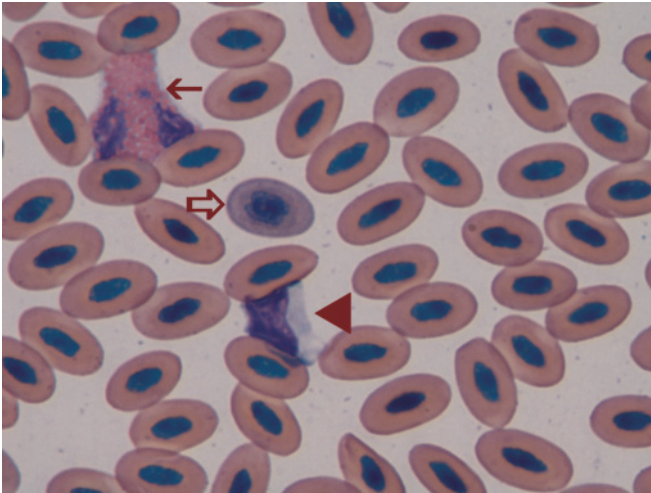


FIGURE 124.6 Broadwing hawk (*Buteo platypterus*): polychromatophilic erythrocyte, open-arrow; eosinophil, solid-arrow; lymphocyte, arrowhead. Wright stain; 100× objective.

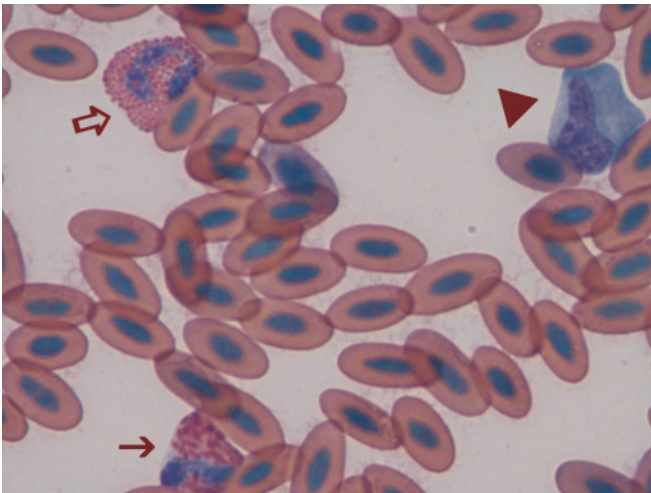


FIGURE 124.7 Red-tailed hawk (*Buteo jamaicensis*): heterophil, solid-arrow; eosinophil, open-arrow; monocyte, arrowhead. Wright stain; 100× objective.

more voluminous gray-blue cytoplasm that occasionally contains vague small eosinophilic granules and vacuoles. These cells are often mistakenly identified as lymphocytes.

Smith and Bush found the WBC values of normal birds of prey were relatively close, with eagles having the highest values (Table 124.3).³² In most raptorial species, the heterophil is the most common leukocyte, but the lymphocyte appears to be the most common cell type in owls.¹⁷ Increases in heterophils may be seen during episodes of stress, infections, and exposure to cold.¹⁴ Increases in basophil and eosinophil counts may reflect intestinal parasitism or hemoparasites.^{15,17}

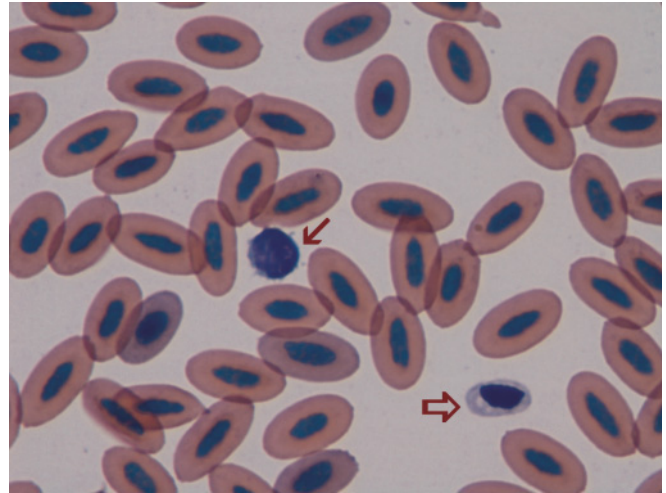


FIGURE 124.8 Barn owl (*Tyto alba*): lymphocyte, solid-arrow; thrombocyte, open-arrow. Wright stain; 100× objective.

Thrombocyte Morphology

The thrombocytes are small, rounded cells that contain a clear reticulated cytoplasm which may contain small red granules or vague vacuolation. Their nuclear:cytoplasmic ratio (N:C) is high and nuclear chromatic staining is dense. Features helpful in distinguishing thrombocytes from lymphocytes are the presence of clear cytoplasm, vacuoles, and granules in the former (Fig. 124.8).⁴ Thrombocytes can also be selectively stained with a periodic acid-Schiff stain to aid in differentiation.³⁰

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Hematology of Ratites

ALICE BLUE-McLENDON and ROBERT A. GREEN

Sample Collection
 Reference Intervals
 Comments on Specific Blood Cell Morphology
 Erythrocyte Morphology
 Thrombocyte Morphology

Granulocyte Morphology
 Lymphocyte Morphology
 Monocyte Morphology
 Conclusions

Acronyms and Abbreviations

EDTA, ethylenediaminetetraacetic acid; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume, RBC, red blood cell; WBC, white blood cell.

Evaluation of the ratite hemogram is important in the veterinary care of diseased birds. The usefulness of ratite hematology is limited by technical difficulties similar to other avian species having nucleated erythrocytes and thrombocytes; however, their large blood volume means that ample blood can be obtained easily for evaluation. The value of the hemogram in confirming health and evaluating disease is well established in most domestic species. Unfortunately, wide laboratory-to-laboratory differences in reference intervals and sparse clinical information concerning hematologic changes expected with specific ratite diseases frustrate the interpretation of ratite hematology.

The focus of ratite medicine varies from the care of an individual bird to that of flock management. Although a full hemogram is quite useful in the diagnosis of or therapy for an individual sick ratite, partial automated hemograms can also provide useful clinical information in screening for the presence of disease in a flock. To this end, the development of automated laser-based hematologic analyzers provides an inexpensive way to perform hematologic screening of ratite flocks. The authors' experience with the Abbott CELL-DYN 3500 Hematology Analyzer (Santa Clara, CA) is that it accurately determines the hematocrit (based on mean corpuscular volume [MCV] and red blood cell [RBC] count) and the absolute heterophil count. The instrument has difficulty separating the similar-sized

ratite small lymphocytes from thrombocytes. However, once a specific disease problem is identified in a flock, knowing the status of heterophils and hematocrit in individual birds can be quite useful in the treatment and evaluation of the disease in the flock. As their accuracy improves, the automated hematologic analyzers of the future may prove increasingly useful in efficient, economic evaluation of flock problems.

SAMPLE COLLECTION

In larger ratites, blood is commonly collected from the cutaneous ulnar vein located on the ventral surface of the wing. In smaller ratites and ostrich chicks, the jugular vein is the preferred site for venipuncture. Blood collection is facilitated in large ratites by using a 21-gauge butterfly needle attached to a 12 inch (30.5 cm) flexible plastic infusion set. This allows some movement of the minimally restrained patient after venipuncture without dislodging the needle and causing hematoma formation. After sufficient blood has been aspirated into the syringe, any bubbles in the infusion line are cleared and the needle is inserted into appropriate Vacutainer tubes. Blood is allowed to flow into the Vacutainer tube without pressure on the syringe. Covering the eyes of ratites, particularly ostriches, by the use of a head hood facilitates their restraint. This technique may initially cause some degree of

excitement or struggling, which compromises interpretation of the hemogram values.

The preferred anticoagulant in ratite blood is citrate (blue top Vacutainer tube). When comparing citrate values to other values, the effect of 1:9 citrate dilution must be considered.⁷ Heparin interferes with staining and causes marked heterophil clumping.² There is increased scatter of the ostrich heterophil population collected in heparin compared with the rather homogeneous heterophil population when collected in citrate (Fig. 125.1). Ethylenediaminetetraacetic acid (EDTA) adversely affects blood from some ratites, causing severe hemolysis or poor cell preservation. It is recommended that blood smears be made immediately after collection for optimal morphology of ratite blood cells. Some laboratories have found that smears made by the coverslip method have fewer smudge cells.⁴

The same laboratory hemogram techniques are used with ratite blood as with other avian species and will not be repeated here.³ If automated hematology analyzers can accurately determine the absolute heterophil count in ratites, then that count along with the manual differential could be used to establish the white blood cell (WBC) count. This would replace the inaccurate and labor-intensive absolute granulocyte count using the eosinophil Unopette (Becton-Dickinson, Rutherford, NJ) and the hemocytometer. The variable spectrophotometric effects of avian erythrocyte nuclei can invalidate automated hemoglobin measurement.

REFERENCE INTERVALS

Reference intervals for ratites are sparse and vary considerably from laboratory to laboratory (Tables 125.1 and 125.2). In part, this reflects the high laboratory error inherent in manual granulocyte count and the common 100-cell count differential, which are used to estimate the total WBC count. Automated hematology analyzers have greatly improved the accuracy of counting and sizing ratite RBCs.

Because ratite hematocrits are similar to other species, there is an inverse relationship between their large RBC size (MCV greater than 175 fL) and their low RBC counts (less than 2–3 10⁶/μL). There are age-related differences in reference interval values, particularly with respect to erythrocyte parameters. Ostrich chicks (1–3 months of age) have hematocrits of about 30%, whereas adult ostriches have hematocrits of about 40%.⁸ Despite the lower MCV of juvenile ostriches, their higher RBC count makes their hematocrit approach that of adult ostriches. In contrast to reports suggesting that the presence of a nucleus in the avian erythrocyte causes lowered mean corpuscular hemoglobin concentration (MCHC), most ratite reference interval MCHC determinations appear quite similar to those of mammals (Tables 125.1 and 125.2).¹¹ In ratite patients, the presence of lipemia or incomplete removal of erythrocyte nuclei causes spuriously increased hemoglobin values (which

**CELL-DYN 3500
(AVIAN WBC DIFFERENTIATION)
CITRATE VS HEPARIN EFFECTS**

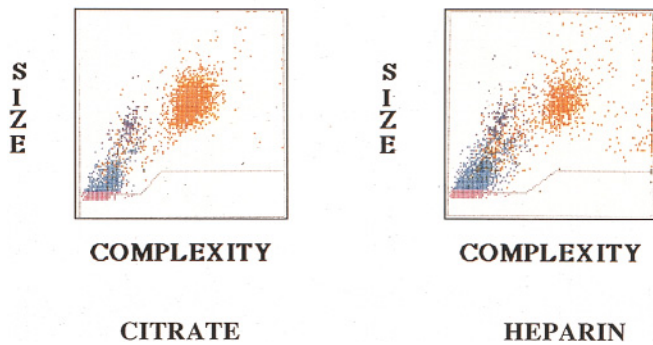


FIGURE 125.1 CELL-DYN 3500 outputs of ostrich blood collected in either sodium citrate or heparin anticoagulants. Note the uniformity of the (orange-colored) heterophil population in citrate compared with the scattered, more diffuse heterophil population when collected in heparin.

TABLE 125.1 Ostrich Reference Intervals

	<i>Fudge 1995⁴</i>		<i>Green 1999⁵</i>				<i>Levi 1989⁸</i>			
	Adults		Adults (>5 yrs)		5–6 months		1–6 yrs		9 months	
	Mean	Range	Mean	SD	Mean	SD	Mean	SD	Mean	SD
WBC (×10 ³ /μL)	18.65	10–24	19.8	6.9	29.2	7.4	5.20	1.60	7.5	1.5
Heterophils (×10 ³ /μL)	13.60	10.8–16.6	15.3	6.6	21.6	6	3.31	0.24	4.57	4.77
Lymphocytes (×10 ³ /μL)	4.51	2.2–7.6	3.1	0.7	4.8	2.4	1.41	0.24	2.66	2.03
Monocytes (×10 ³ /μL)	0.49	0–0.7	0.7	0.4	1.3	1.2	0.10	0.05	0.25	0.11
Basophils (×10 ³ /μL)	0.04	0–0.4	0.3	0.2	0.8	0.5	0.01	0.02	0.01	0.02
Eosinophils (×10 ³ /μL)	0.01	0–0.4	0.2	0.2	0.3	0.3	0.01	0.02	0.015	0.03
RBC (×10 ⁶ /μL)	1.80		1.5	0.2	1.7	0.1	1.5	0.2	2.1	0.3
Hematocrit (%)	45	41–57	35	3.6	33.8	2.8	40	4	37	3
Hemoglobin (g/dL)	16.9		11.7	1.4	10.8	0.9	13.8	3	12.7	2.1
MCV (fL)	212		219	5.3	192	8	193	52	164	16
MCHC (g/dL)	37.65		33	2.6	32	0.01	37	4	35	6
RDW (g/dL)	11.11		11.1	1.3	12.1	0.7	—	—	—	—

TABLE 125.2 Ratite Reference Intervals

	Emu		Cassowary		Rhea	
	<i>Fudge 1996^d</i>		<i>Stewart 1994^a</i>		<i>Green 1999^b</i>	
	Adults		Age not given		1 yr	
	Mean	Range	Mean	S.D.	Mean	S.D.
WBC ($\times 10^3/\mu\text{L}$)	14.87	8–21	18	4.50	13.6	3.3
Heterophils ($\times 10^3/\mu\text{L}$)	11.72	8.0–13.1	13.99	4.64	8.1	2.7
Lymphocytes ($\times 10^3/\mu\text{L}$)	2.94	1.5–6.5	3.546	1.87	4.3	1
Monocytes ($\times 10^3/\mu\text{L}$)	0.01	0–0.2	0.432	0.43	0.5	0.5
Basophils ($\times 10^3/\mu\text{L}$)	0.03	0–0.2			0.5	0.4
Eosinophils ($\times 10^3/\mu\text{L}$)	0.38	0–0.9			0.2	0.2
RBC ($\times 10^6/\mu\text{L}$)					2.25	0.1
Hematocrit (%)					41.6	1.9
Hemoglobin (g/dL)					13.4	0.45
MCV (fL)					185	5.9
MCHC (g/dL)					32	0.01
RDW ^a (g/dL)					12.6	0.6

^aRDW is RBC distribution width determined by Abbott CELL-DYN 3500 analysis.

also increase MCH or MCHC). It is noted that the WBC count of juvenile ostriches is higher than that of adults, so heterophilic leukocytosis must be interpreted with caution in young ratites.^{4,8} Some reference ranges show higher lymphocyte and basophil counts in young ostriches than in adults.

COMMENTS ON SPECIFIC BLOOD CELL MORPHOLOGY

Erythrocyte Morphology

The ratite erythrocyte is an oval nucleated cell that is much larger (MCV > 200 fL) than most other avian species (MCV = 125–179 fL) or common domestic animals (MCV = 40–70 fL). It has a centrally placed compressed oval nucleus with uniformly clumped chromatin which becomes progressively more condensed as cells age. The nucleus of the ostrich erythrocyte varies in shape from an elongated oval to teardrop-shape. The erythrocyte cytoplasm has a uniform orange-pink color when stained with most Wright stains.

Young polychromatophilic ratite erythrocytes normally comprise approximately 1–2% of the total erythrocyte population and reflect the status of marrow erythrocyte production. Immature ratite erythrocytes have a more round shape than mature erythrocytes, and their cytoplasmic color ranges from blue to gray-purple (Fig. 125.2). Their nucleus is also more round and much less condensed than the mature erythrocyte nucleus. Reticulocyte counts are uncommonly performed on ratite blood but can be used in quantifying regenerative responses similar to other avian species. Using Wright stain, reticulocytes tend to be slightly larger and bluer (polychromatophilic macrocytes) than mature erythrocytes. The new methylene blue stain reveals variable numbers of dark blue staining RNA aggregates that surround the reticulocyte nucleus. Both

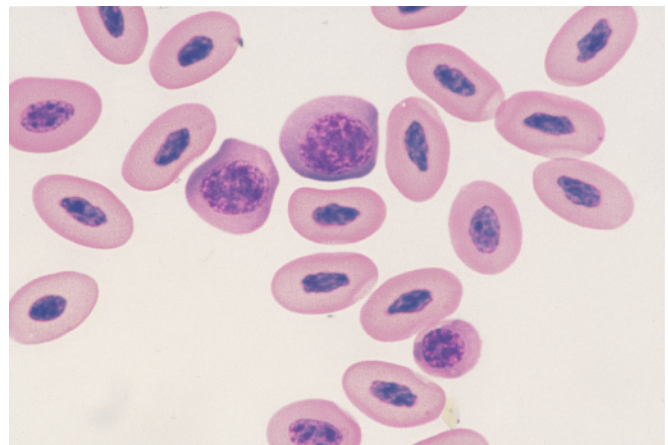


FIGURE 125.2 Diff-Quik stain of emu blood showing immature erythrocytes. Note the two larger rubricytes and the smaller metarubricyte having blue-gray (polychromatophilic) cytoplasm with nuclei that are more round and have less condensed nuclear chromatin than the mature erythrocytes.

punctate and aggregate reticulocytes are present in ratites, but only the aggregate forms are counted in performing a reticulocyte count (similar to the evaluation of feline reticulocytes).³

Thrombocyte Morphology

The ratite thrombocyte is a nucleated cell that is about half the size of their erythrocytes. It has a round to oval shape, with a dense pyknotic round nucleus and a moderate amount of clear, somewhat reticulated cytoplasm. The cytoplasm often has several reddish granules and may have several clear spaces or vacuoles (which are more commonly noted in partially activated thrombocytes) (Figs. 125.3A and 125.4). Thrombocytes often

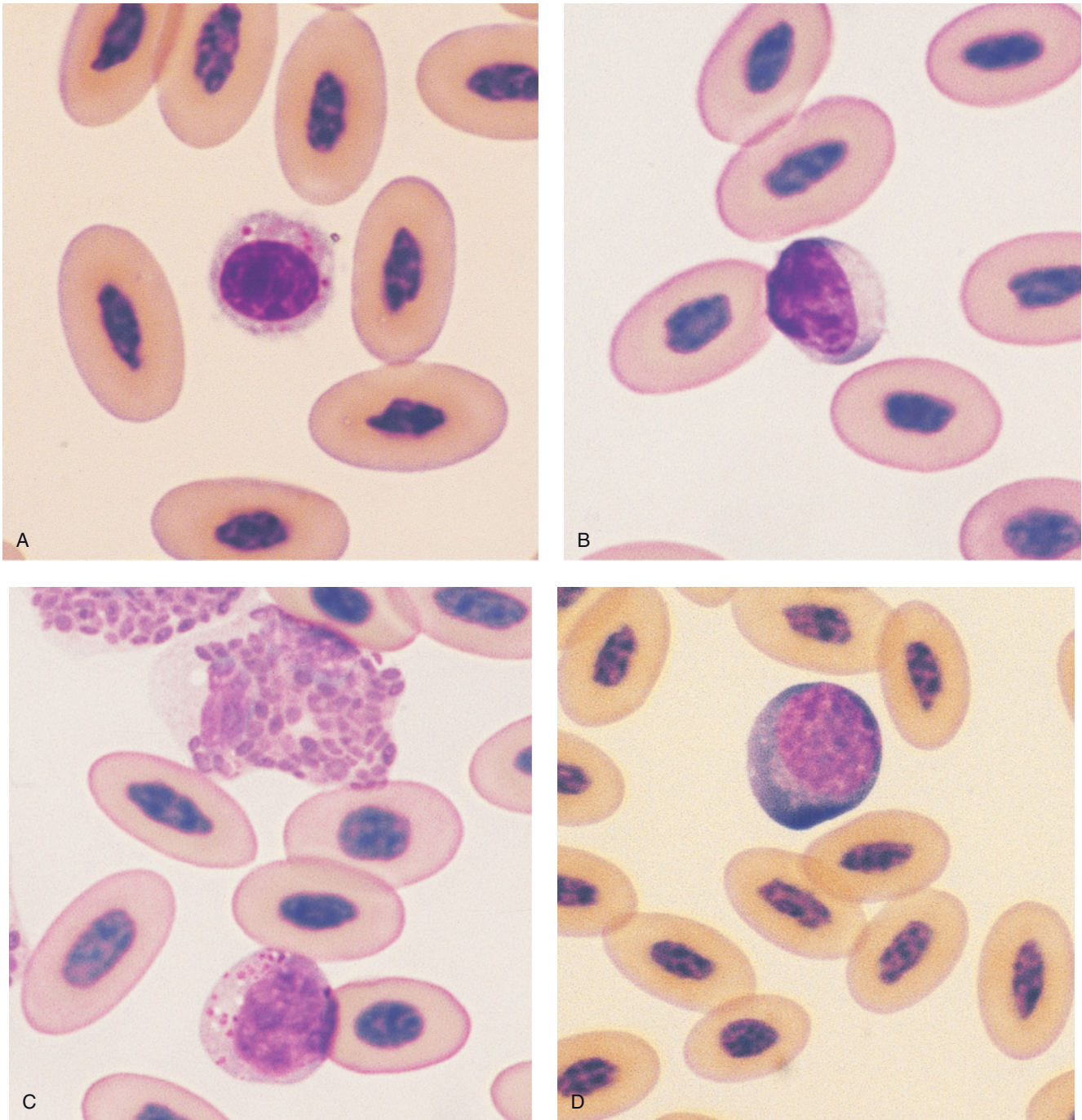


FIGURE 125.3 (A) Typical ostrich thrombocyte showing a few red granules in its cytoplasm with a centrally located, highly condensed oval nucleus. (B) Small ostrich lymphocyte showing a less condensed, indented eccentric nucleus with light blue cytoplasm. (C) A heavily granulated heterophil (top) with an immature thrombocyte (bottom) having granules similar in appearance to the mature thrombocyte and a slightly enlarged, moderately condensed oval nucleus. Alternatively, this cell may be a granulated lymphocyte, although its granule and nuclear characteristics seem more consistent with a thrombocyte classification. (D) A plasma cell (or reactive lymphocyte) characterized by a round eccentric nucleus with dark blue cytoplasm and a prominent Golgi zone adjacent to the nucleus.

tend to aggregate in clusters of reactive thrombocytes on blood smears, which makes estimating the thrombocyte count difficult. Although not well established in ratites, normal thrombocyte counts range from 20,000 to 30,000/ μL or 10–15 per 100 erythrocytes on blood smears. A qualitative thrombocyte count is often deter-

mined during blood smear evaluation and is usually reported as decreased, adequate, or increased. Adequate thrombocyte numbers are usually associated with finding 5–10 thrombocytes per five oil immersion fields on a blood smear. Immature thrombocytes are occasionally noted on smears, indicating enhanced thrombocyte

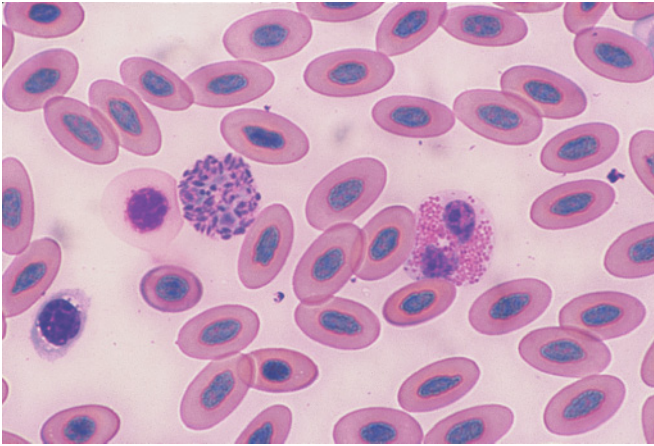


FIGURE 125.4 Comparison of the Diff-Quik stain appearance of the ostrich eosinophil (right) having a bilobed nucleus and numerous small round red granules with that of a heavily granulated heterophil (center). A thrombocyte (lower left) with a centrally located oval nucleus and several perinuclear clear zones is also present.

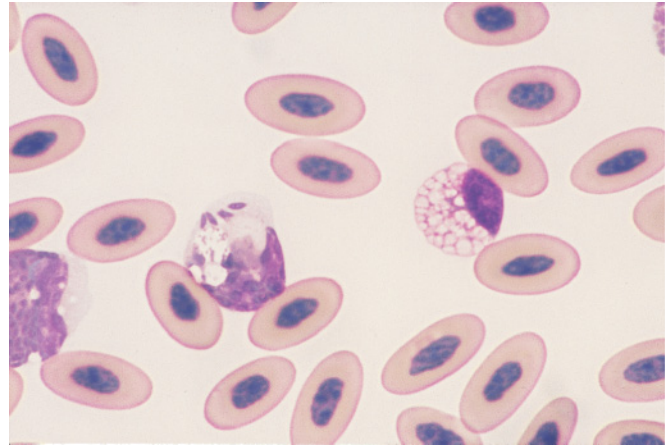


FIGURE 125.5 Diff-Quik stain of ostrich blood showing a basophil (right) with an eccentric nucleus and a vacuolated appearance due to lysis of its granules. Compare this cell with the toxic heterophil (left of center) characterized by cytoplasmic hypogranularity and vacuolization.

production. Their size is somewhat larger than mature thrombocytes (approaching the size of a large lymphocyte). Their nuclear shape is round, with a condensed clumped chromatin pattern; their cytoplasm is lightly basophilic with increased numbers of small, round, red granules, similar to those found in mature thrombocytes (Fig. 125.3C).

Recent studies using aspirin to inhibit normal thrombocyte aggregation in whole blood from ostriches suggested that prostaglandin-dependent mechanisms play a role in thrombocyte activation, similar to mammalian platelets.¹⁰ Agonists causing irreversible whole blood thrombocyte aggregation included platelet-activating factor and collagen, but not adenosine diphosphate (ADP). Hemorrhagic diseases of ratites caused by abnormal thrombocyte function are uncommon; however, thrombocyte dysfunction may be responsible for a high incidence of hemorrhage noted in emus with hereditary gangliosidosis.¹

Granulocyte Morphology

Heterophils are the most numerous WBCs in ratite blood and have similar functions to those of neutrophils in mammalian blood. The mature heterophil is a round cell with a segmented nucleus that is often partially occluded by numerous round to fusiform granules that have a red-orange color (Figs. 125.3C and 125.4). The background cytoplasm is clear in the mature heterophil. Immature heterophils are uncommon in normal ratite blood, and their stage of maturation is based on nuclear shapes, similar to other species. The cytoplasm of immature heterophils is slightly more basophilic than that of mature heterophils.

The granulation of immature heterophils ranges from early cells with moderate numbers of basophilic round (primary) granules, to intermediate cells having mostly round eosinophilic granules, to mature cells

having mostly orange fusiform granules. Increased immature heterophils in blood (called a left shift) imply increased demand for heterophils, often associated with inflammatory diseases of ratites. In diseases associated with toxicity, it is common to see abnormal heterophil granulation characterized by cells that are poorly granulated or retain the round primary granules of early precursors (Fig. 125.5). In severe toxicity, heterophils may partially degranulate, nuclear swelling occurs, and their cytoplasm becomes more basophilic.³ An Abbott CELL-DYN output from an ostrich with septicemia shows the effect of increased immature heterophils by causing the heterophil population pattern to be extended upward compared with the control (Fig. 125.6). Inflammatory diseases of ratites associated with heterophilic left shifts and toxic changes are usually associated with a poor prognosis.

Eosinophils are found in low numbers in blood from normal ratites, and their function is uncertain. The emu reference interval suggests slightly higher levels of eosinophils in emus than ostriches (Table 125.2). Eosinophils often have a bilobed nucleus with abundant small, round, red-to-pink granules and light blue cytoplasm (Fig. 125.4). The granules of eosinophil precursors are larger, and primary granules may also be present in their cytoplasm. Parasitism appears to induce eosinophilia less commonly in ratites than in mammals or certain other avian species (particularly raptors).

Basophils are found in low numbers in normal ratite blood (although they are more common than eosinophils in juvenile ostriches). The ratite basophil is slightly smaller than the heterophil and has an eccentric round to oval, nonsegmented nucleus. The basophil cytoplasm is moderate in amount and often has a distinct purple hue. The numerous dark metachromatic granules of the basophil commonly dissolve with routine Wright stain, leaving the cytoplasm with a vacuolated or reticulated appearance (Fig. 125.5). A few

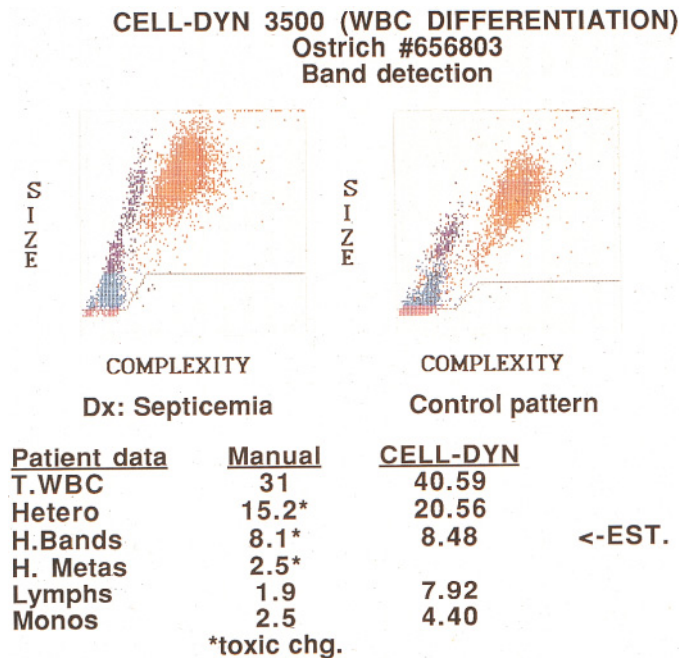


FIGURE 125.6 CELL-DYN 3500 patterns for WBC collected from a normal ostrich (right) and from an ostrich with septicemia (left). Note the orange-colored heterophil population is shifted upward and to the left, indicating the presence of immature (larger, less complex) heterophils in the septicemic ostrich. Monocytes are indicated by purple, lymphocytes by blue, and background erythrocytic debris with thrombocytes by red. The CELL-DYN analysis of ratite lymphocyte populations is consistently overestimated because of interference from similar-sized thrombocytes. Toxic heterophils (noted on the blood smear) featured hypogranularity that also produced a leftward shift in the CELL-DYN heterophil population. The heterophil band population was estimated (EST.) by the CELL-DYN analysis and appears to correlate well with the left shift noted in the manual count.

remnant basophilic granules are often present to assist cell classification. Although basophil function is unclear in ratites, basophilia is occasionally noted in hypersensitivity reactions and chronic respiratory diseases.

Lymphocyte Morphology

Lymphocytes are the second most common WBC in normal ratites, and lymphopenias are commonly associated with diseases causing increased stress. Lymphocyte morphology is similar to that of other avian species, with cells varying in size from small lymphocytes (which may be difficult to differentiate from thrombocytes) to intermediate or large lymphocytes (which may become reactive and appear similar to monocytes). The nuclear shape of small lymphocytes is round with a lacy chromatin pattern. Their cytoplasm varies in amount from scant to moderate and is usually lightly basophilic (Fig. 125.3B). This feature is quite helpful in distinguishing small lymphocytes from activated thrombocytes having clear cytoplasm. Lymphocytes are often found abutting adjacent eryth-

rocytes, which makes their shape appear rectangular. Degenerate lymphocytes may have scant cytoplasm or cytoplasmic blebs. Occasionally, larger lymphocytes are noted with a few azurophilic granules, similar to those found in killer lymphocytes of mammals. Plasmacytoid or reactive lymphocytes may be seen in ratites, particularly during convalescent immune responses (Fig. 125.3D). These plasma cell-like lymphocytes have an eccentric round nucleus, dark basophilic cytoplasm, and a pale perinuclear Golgi zone. Mild lymphocytosis is also associated with immune stimulation, noted particularly during convalescence. Marked lymphocytosis of either small or larger lymphoid cells is suggestive of lymphocytic leukemia in ratites.⁶

Monocyte Morphology

Monocytes are found in low numbers in ratite blood, and their morphology is quite similar to mammalian monocytes. They are large cells with moderate amounts of blue-gray cytoplasm that occasionally has small discrete vacuoles. Their nuclei are pleomorphic with a chromatin pattern that is lacy and somewhat less condensed than lymphocytes. Distinct cytoplasmic granules are uncommon in ratite monocytes, although faint, dust-like eosinophilic granules are noted commonly. Differentiating monocytes from enlarged reactive lymphocytes can be challenging in ratite blood smears. Monocytosis is associated with various chronic granulomatous diseases of ratites, including mycoses, and diseases in which marked tissue necrosis occurs.

CONCLUSIONS

In the 1990s when individual ratites were valued at several thousand dollars, it was easy to justify veterinary care and extensive laboratory evaluation of individual birds. Despite this, there was a paucity of veterinary literature available concerning hematologic changes expected with ratite diseases. Hematologic evaluation can provide useful information with respect to medical evaluation of diseased ratites, no matter the economic state of the ratite industry.

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Hematology of Fishes

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Hematologic Methods

- Collecting a Blood Sample
- Determination of Cell Counts
- Hemoglobin Determination

Blood Cell Formation and Function

- Blood Cell Formation
- Blood Cell Morphology and Function
- Blood Coagulation
- Blood Values in Fishes

Acronyms and Abbreviations

AGM, aorta-gonad-mesonephros; APTT, activated partial thromboplastin time; CBC, complete blood count; EDTA, ethylenediaminetetraacetic acid; Hgb, hemoglobin; ICM, intermediate cell mass; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCHMS-222, Tricaine methanesulfonate; MCV, mean corpuscular volume; PAS, periodic acid-Schiff; PT, modified prothrombin time; RBC, red blood cell; RVV-T, Russell viper venom time; WBC, white blood cell.

Historically, the field of fish hematology has spanned the past century as reviewed in several publications;^{1,10,13,21} however, the older literature is often confusing and conflicting. Successful development of hematology as a diagnostic tool in fishes has only occurred in the past 20 years, as information concerning the function and maturation of blood cells, physiologic responses, and standardized hematologic techniques became available.

A number of factors make diagnostic hematology more challenging in fishes than in mammalian species. First, a review of the literature presents inconsistencies in the nomenclature, description, differentiation, maturation, and function of fish blood cells. Second, nucleated red blood cells (RBCs) and leukocytes overlap in size, preventing the use of automated methods to determine cell counts.²⁸ Lastly, the number of fish species and their diversity in morphologic form and ecologic function makes generalizations about the group difficult. The generic term “fish” includes primitive jawless vertebrates, vertebrates with cartilaginous skeletons, as well as more advanced species having bony skeletons. Bony fishes have more in common with and are more closely related to other bony vertebrates (e.g. mammals) than they are to their cartilaginous counterparts. In

order to focus the discussion of this highly diverse set of animals, this chapter will be limited to bony fishes (Class Osteichthyes) that possess many similarities in blood cell morphology and function with other bony vertebrates.

HEMATOLOGIC METHODS

Hematologic techniques developed for mammalian species are generally applicable for fishes with slight modification. Most fishes respond adversely to being handled. Sedation or anesthesia before bleeding is recommended to decrease hematologic changes from the stress of restraint. A number of anesthetic agents can be effectively used with fishes. MS-222 at 100–200 mg/L is the most widely accepted, and is presently the only FDA approved anesthetic for finfish. MS-222 should be buffered with sodium bicarbonate or other suitable buffer to prevent the extreme decrease in pH of unbuffered MS-222 from causing significant alterations in physiologic and hematologic measurements. The anesthetic solution should be of sufficient concentration to induce stage 2 anesthesia (i.e. loss of equilibrium) within 20 seconds, and stage 3 anesthesia within 1

minute. The fish is bled before respiratory depression induces significant hypoxia. Anesthesia induced hypoxia can be minimized by using an aerated anesthetic solution.

Collecting a Blood Sample

Fishes can be bled from a number of sites; the most common being the caudal tail vessels. Other sites include the cuvarian duct (a vessel within the oral cavity), branchial vessels, and bleeding directly from the heart. Cardiac puncture is traumatic and should not be performed on a fish that is to be recovered after blood collection. The cuvarian duct and branchial vessels can only be sampled in large fish. Severing the tail or cutting through the musculature to expose vessels should not be used to collect blood as the sample will be contaminated with tissue fluid or other cellular elements.

Only good quality blood samples should be used for hematological evaluation. Clotted, clumped, hemolyzed, or improperly stored samples should be discarded. Fish blood hemolyzes easily and best collection results are obtained with a small (1–3 mL) syringe. Larger blood samples can successfully be collected with a vacutainer system. Blood should be transferred immediately to a blood tube containing anticoagulant to minimize clotting. Pediatric-sized tubes (0.5 mL) are an ideal size for most fishes. The choice of anticoagulant for preservation of cellular morphology appears to be species specific. Salmonid, cyprinid, and sturgeon blood cells retain morphology best with heparin, while catfish, bass, tilapia, and pacu cells preserve best in ethylenediaminetetraacetic acid (EDTA).^{22,25,27,33,50} Occasionally, blood will clot in the presence of anticoagulant. Clots may be grossly visible, or thrombocytes will appear clumped when the blood film is viewed under the microscope. Use of a different anticoagulant and reducing pre-capture stress may prevent thrombocyte clumping.

Determination of Cell Counts

Reliability of the manual complete blood counts (CBCs) is one of the greatest difficulties for diagnostic hematology in fishes. Counts must be determined manually as automated methods cannot distinguish erythrocytes from leukocytes due to the overlapping size of these cells. Sources of error associated with manual counting can occur when diluting and mixing the blood, filling the hemacytometer, and during identification and counting of cells. Misidentification frequently happens when distinguishing activated thrombocytes and lysed RBCs from leukocytes. Thrombocytes are usually oval in shape but become small and round similar to a lymphocyte when activated. When erythrocytes lyse in the blood diluent, the nucleus takes up stain similar to a leukocyte. Lysed erythrocytes can be distinguished from leukocytes with practice; however, it is impossible to consistently differentiate leukocytes from thrombo-

cytes in the hemacytometer. Leukocytes and thrombocytes are best separated and identified during the differential count on a stained blood smear.

Manually counting cells on a hemacytometer can be time consuming but provides the greatest accuracy for a CBC in fishes. A diluent should be used that differentially stains RBCs and leukocytes. Natt-Herrick's,⁴² Rees-Ecker,³⁷ or modified Dacie's² diluents are effective with most fish species. Depending on the concentration of cells in the blood, a 1:100 or 1:200 dilution of blood to filtered diluent is recommended. Allow the cells to stain for 2–4 minutes before filling the hemacytometer.

As in mammals, RBCs are counted in five secondary squares of the center primary square. The raw count, multiplied by 5000 for a 1:100 dilution or by 10,000 for a 1:200 dilution, will give the number of erythrocytes per microliter of blood. The leukocyte count is frequently higher in fishes than in mammals, and leukocytes and thrombocytes are counted only in the four corner primary squares. The raw count, multiplied by 250 for a 1:100 dilution or by 500 for a 1:200 dilution, will give the number of cells per microliter of blood. This provides a combined total count for leukocytes and thrombocytes. Thrombocytes are then enumerated during the differential count on a stained blood smear.

Differential counts should be determined from good quality blood smears. Direct smears made without anticoagulant often have thrombocyte clumping which affects cell distribution and cell counts. Scan each slide before beginning the differential count to check the quality of the smear and to become familiar with the cell types present and appearance of the smear. Count leukocytes and thrombocytes until 200 leukocytes are enumerated in the monolayer portion of the blood smear. Thus, all cell types are counted except RBCs until the sum of the lymphocytes, monocytes and various granulocytes totals 200, regardless of the number of thrombocytes encountered. As thrombocytes frequently make up greater than 50% of the cells, total counts greater than 400 are common. The percentage of thrombocytes is then subtracted from the combined leukocyte-thrombocyte count determined on the hemacytometer to give the total leukocyte count and the thrombocyte count. The percentage of each leukocyte type is multiplied by the combined leukocyte-thrombocyte count to give the absolute number of each cell type.

Hemoglobin Determination

Hemoglobin is best determined using the standard cyanomethemoglobin method. Test solutions should be centrifuged before measuring the absorbance as nucleated RBCs release a flocculent material upon lysis. The released flocculent precipitate interferes with absorbance readings unless removed by centrifugation. The erythrocyte indices mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) are calculated as in mammals.

BLOOD CELL FORMATION AND FUNCTION

Blood Cell Formation

Blood cells present in the blood of fishes vary with the species but include: RBCs, thrombocytes, lymphocytes, monocytes, neutrophils (heterophils), eosinophils, basophils, and immature forms.^{10,13,57} Hematopoiesis in fishes is similar to that in mammals and occurs in two waves, primitive hematopoiesis and definitive hematopoiesis. In many fish species, development in the egg stage is brief with free swimming larvae hatching 2–3 days after fertilization. Further development including organogenesis and maturation of tissues into adult form and function occurs over the next 4–8 weeks. Primitive hematopoiesis begins while still in the egg stage or soon after hatching. Cells lying lateral to the somites in the developing larva begin to express markers for both vascular and blood elements. These cells migrate medially to form the intermediate cell mass (ICM) ventral to the notochord.⁹ The ICM is equivalent to the blood islands in the mammalian yolk sac, and the cells differentiate into endothelial cells, trunk vasculature, and proerythroblasts.⁹ Some fish species utilize blood islands in the yolk sac as the site of primitive hematopoiesis, while others, such as trout, utilize both the yolk sac and the ICM.¹⁷ Proerythroblasts from these primitive hematopoietic centers enter the circulation and mature into primitive RBCs which are distinct from adult RBCs, having less cytoplasm and an elongated nucleus. Primitive RBCs contain hemoglobin and undergo globin switching from primitive embryonic chains to adult chains as do mammals.

A second site of primitive hematopoiesis, called the rostral blood islands, forms in the anterior mesoderm of developing fish. This region produces both myeloid cells and vascular elements. Myeloid cells express *pu.1*, a marker for macrophages and granulocytes. Subsets of these cells also express *l-plastin* and *mpo* that are markers for macrophages and granulocytes respectively.⁹ These early phagocytes migrate from the rostral blood islands across the yolk sac and also enter the circulation. They are phagocytic and migrate to sites of infection.⁹ It is not known if primitive and definitive phagocytes are produced, similar to RBCs.

Adult hematopoietic stem cells are produced in a region of the developing fish called the aorta-gonad-mesonephros (AGM). In mammals, these hematopoietic stem cells translocate to the fetal liver and bone marrow. In fishes, these cells translocate to the kidney with progenitors of all blood cell types present in the kidney tissue.⁴³ In some marine species, cells from the AGM also translocate to the spleen which then becomes the primary site of erythropoiesis and myelopoiesis.¹⁵ In the developing fish, the kidney is first predominately erythropoietic. Erythroid precursors are similar to those seen in mammals and are released from the kidney into circulation at an immature stage. Reticulocytes are frequently present in fish blood preparations. Later in development of the kidney, myelopoiesis dominates

and finally the kidney becomes predominately lymphopoietic. Fish lymphocytes can be divided into T, B, and nonspecific cytotoxic lymphocytes.^{7,11} B cells express immunoglobulin while T cells express the T cell receptor. T cells develop and mature in the thymus, while B cells develop in the pronephros region of the kidney.¹⁷ The origin of lymphocyte progenitors that seed the thymus has not been determined, but *Ikaros* (a hematopoietic transcription factor) positive cells in the AGM probably translocate to the thymus.

Thrombocytes are present in circulation early in development. However, the cell equivalent to the megakaryocyte, the maturation stages, and primary site of thrombopoiesis have not been identified.

Blood Cell Morphology and Function

As in mammals, RBCs contain hemoglobin and transport oxygen, albeit at much lower ambient oxygen concentrations. Fish RBCs are nucleated, are more metabolically active than their mammalian counterparts, and may play a significant role in modulating blood chemistry.²⁴ Reticulocytes, easily recognized by their slightly basophilic cytoplasm, are smaller cells with a more open nucleus.

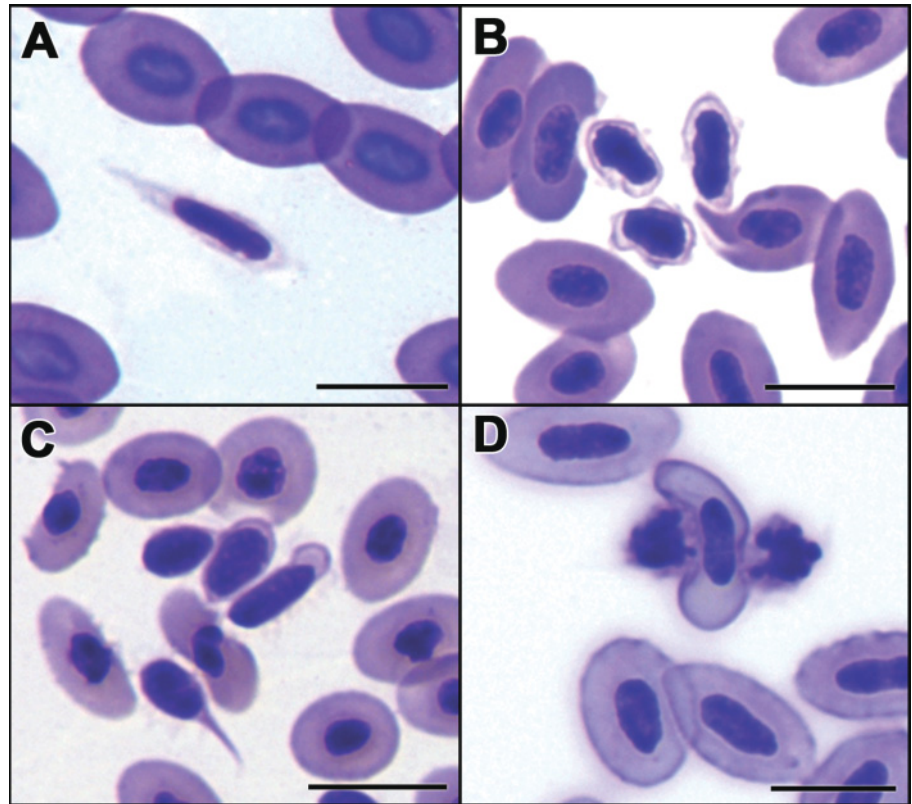
Thrombocytes are involved in coagulation and clot formation. Ultrastructurally, the cells are similar to platelets, with an interconnecting canalicular system and cytoplasmic granules. Thrombocytes are activated by the same mechanisms as mammalian platelets, and they cross-react with human platelet markers. Thrombocytes have a condensed nucleus with clear or light staining cytoplasm (Fig. 126.1). Cell shape varies with the activation state, changing from spiked or oval to round as the cells activate. In anticoagulated blood, the thrombocyte nucleus may segment or remain oval.

Lymphocytes function in innate and adaptive immune responses with T, B, and nonspecific cytotoxic lymphocytes present. Lymphocytes are usually small cells with dark blue cytoplasm and a condensed nucleus (Fig. 126.2). The cells are round and frequently have pseudopods along the cell margin. As lymphocytes become reactive, they increase in size, the nucleus becomes less condensed, and they may acquire the classic plasma cell morphology.

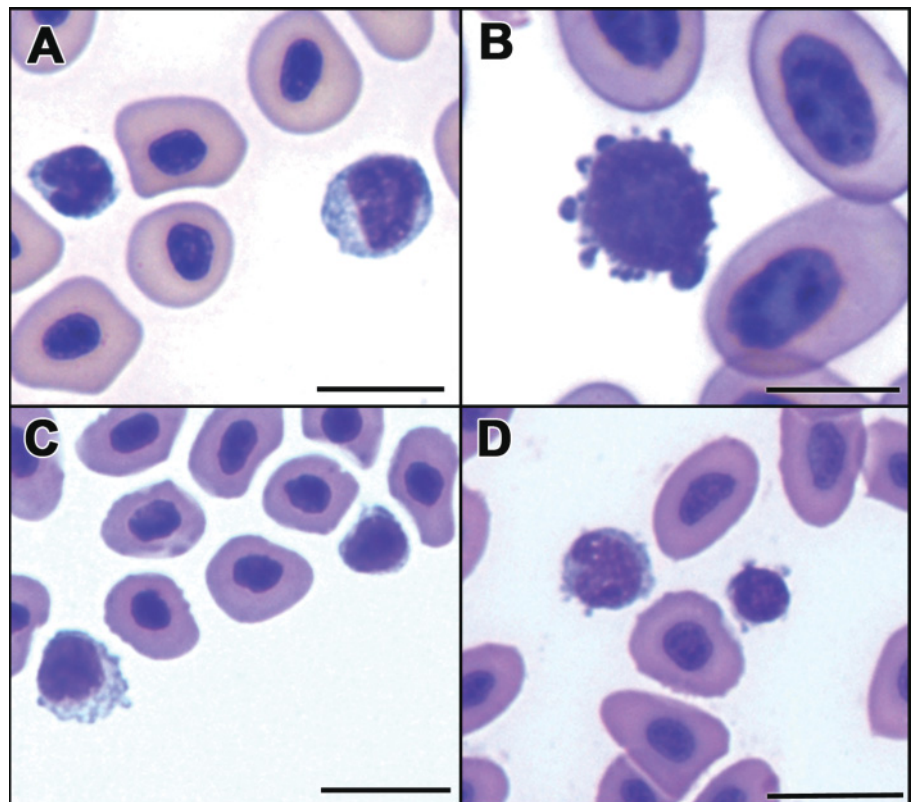
Granulocyte nomenclature and morphology have not definitively been established for fishes and as such there are inconsistencies in granulocyte identification. The terms neutrophil and heterophil are frequently used interchangeably for cells with no visible or very fine cytoplasmic granules. Cells containing reddish cytoplasmic granules of varying sizes and shape have been called both eosinophils and heterophils. Cells with purplish cytoplasmic granules have been called both basophils and heterophils. Many reports do not contain photomicrographs of the cells, making correlation between studies difficult. Until either a consensus is reached regarding cell nomenclature or unique surface

FIGURE 126.1 Thrombocytes.

Thrombocyte morphology and staining can be affected by activation and anticoagulants. Cells have translucent cytoplasm and a condensed nucleus. Scale bar, 10 μm . (A) Pacu – *Colossoma macropomum*, with EDTA; Wright-Giemsa stain. (B) Angelfish – *Pterophyllum scalare*, direct smear, no anticoagulant; Hema 3 stain. (C) Cobia – *Rachycentron canadum*, direct smear, no anticoagulant; Hema 3 stain. (D) Grass carp – *Ctenopharyngodon idella*, with EDTA; Hema 3 stain.

**FIGURE 126.2** Lymphocytes.

Lymphocytes are round cells with scant blue cytoplasm and a dark condensed nucleus; when activated the cell becomes larger with more abundant cytoplasm. Scale bar, 10 μm . (A) Channel catfish – *Ictalurus punctatus*, with heparin; Hema 3 stain. (B) Sturgeon – *Acipenser brevirostrum*, with heparin; Hema 3 stain. (C) Cobia – *Rachycentron canadum*, direct smear, no anticoagulant; Hema 3 stain. (D) Striped bass – *Morone saxatilis*, direct smear no anticoagulant; Wright-Giemsa stain.



markers are identified for each cell type, nomenclature discrepancies will continue.

Granulocytes are one of the largest cells in circulation (Figs. 126.3, 126.4, and 126.5). The cytoplasm is usually a pale blue. In most species, the nucleus is open and oval to kidney bean shaped. In some species, the nucleus is segmented, possibly indicating that the cells mature in circulation before migrating into the tissues. The cytoplasm may have a grainy appearance with no visible granules, or may contain granules of variable size and shape depending on the specific cell type.

Neutrophils are involved in inflammation and the inflammatory response, and are phagocytic. They stain positive for myeloperoxidase and acid phosphatase. Functional differences between fish neutrophils and heterophils have not been determined. Eosinophils function in parasite killing and phagocytosis. Eosinophils stain PAS positive and are acid phosphatase negative. Basophils are rarely reported. It could be that basophils are just present in low numbers, or that their morphology is not well preserved with routine alcohol-based fixatives and stains. In one study basophils were best identified with lead subacetate fixative and acid toluidine blue stain.⁵¹ Little is known about basophil function, but they are thought to be similar to mammalian basophils.

Monocytes are round cells with a round to horseshoe shaped nucleus (Fig. 126.6). The cytoplasm stains a deep blue and is frequently vacuolated. As in mammals, monocytes circulate through the blood, migrate into the tissues and become macrophages. Monocytes and macrophages are the primary phagocytic cell in fishes.^{3,10}

Blood Coagulation

Blood coagulation in fishes is not well studied, but appears similar to mammalian coagulation. The majority of genes regulating coagulation are highly conserved between all vertebrates.³² Substances that aggregate platelets or are vasoactive in mammals cause similar reactions in fishes.¹³ The blood coagulation cascade contains both intrinsic and extrinsic pathways as evidenced by measurement of prothrombin time (PT), activated partial thromboplastin time (APTT), and Russell viper venom time (RVV-T).^{31,55} Fishes also appear to have a similar anticoagulant pathway with the natural anticoagulants protein C, antithrombin, and heparin cofactor II present in their plasma.³¹ Additionally, acute and chronic stress, such as that caused by capture or from production fish management, can cause a thrombocytosis and decrease clotting time.⁶

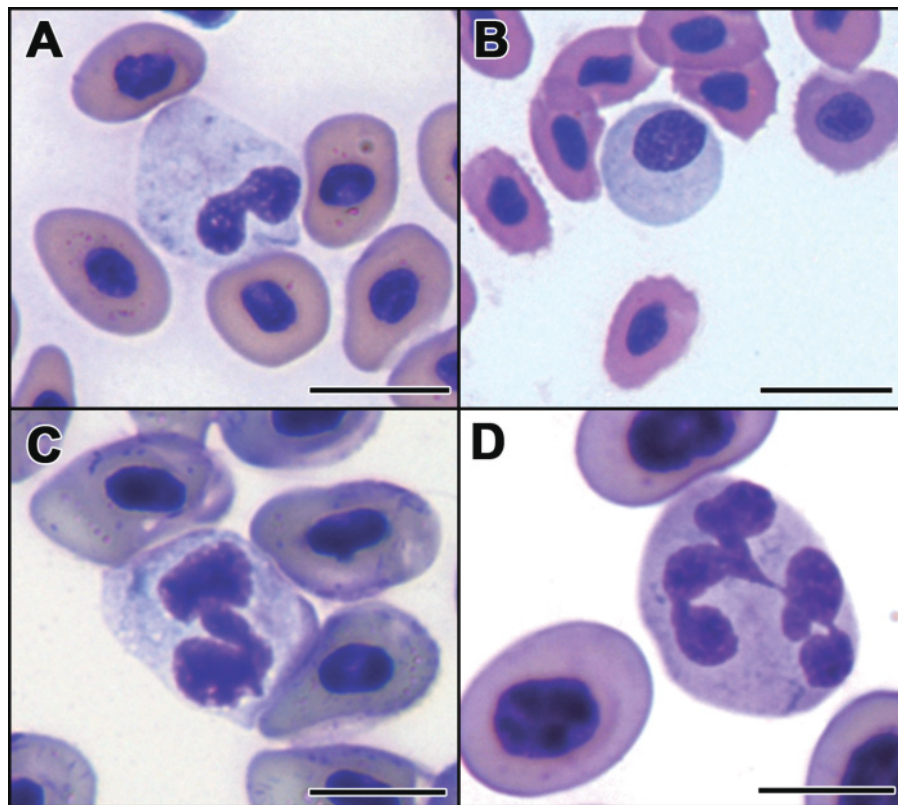


FIGURE 126.3 Granulocytes. Granulocytes with no or finely stained granules; nucleus is oval to segmented. Staining appearance can be affected by anticoagulant. Scale bar, 10 μ m. (A) Catfish – *Ictalurus punctatus*, with heparin; Hema 3 stain. (B) Striped bass – *Morone saxatilis*, direct smear, no anticoagulant; Wright-Giemsa stain. (C) Rainbow trout – *Oncorhynchus mykiss*, with heparin; Hema 3 stain. (D) Sturgeon – *Acipenser brevirostrum*, with heparin; Hema 3 stain.

FIGURE 126.4 Granulocytes.

Granulocytes with large red staining granules; nucleus is oval to segmented. Staining appearance can be affected by anticoagulant. Scale bar, 10 μm . (A) Cobia – *Rachycentron canadum*, direct smear, no anticoagulant; Hema 3 stain. (B) Sturgeon – *Acipenser brevirostrum*, with heparin; Hema 3 stain. (C) Angelfish – *Pterophyllum scalare*, direct smear, no anticoagulant; Hema 3 stain. (D) Smallmouth bass – *Micropterus dolomieu*, with heparin; Wright's stain. (Courtesy of Jesse A. Fallon.)

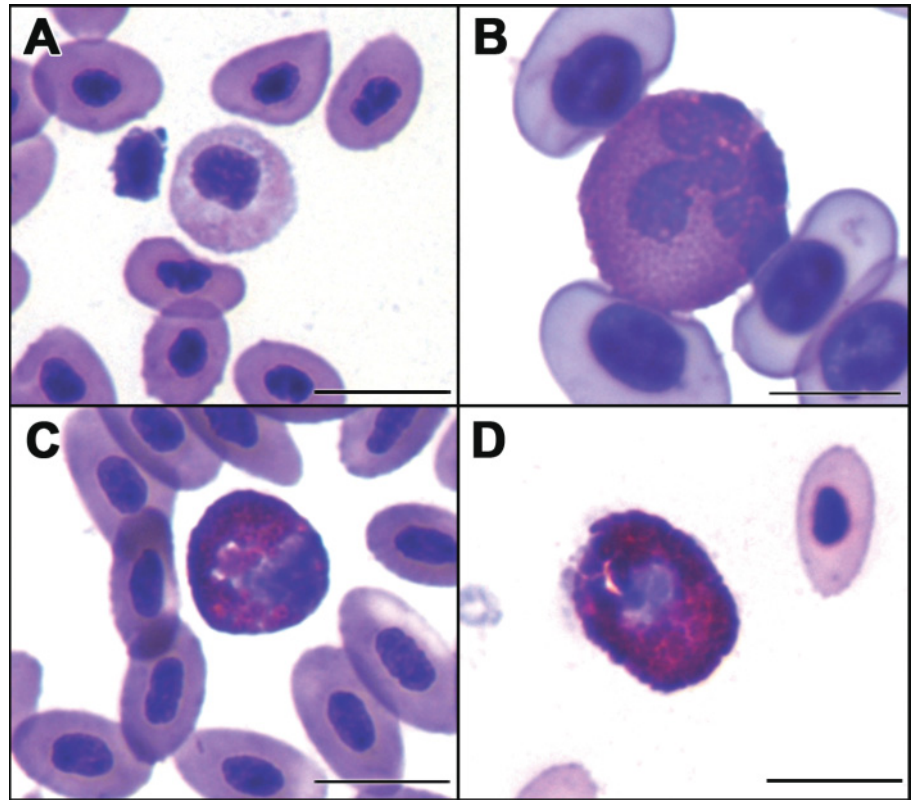
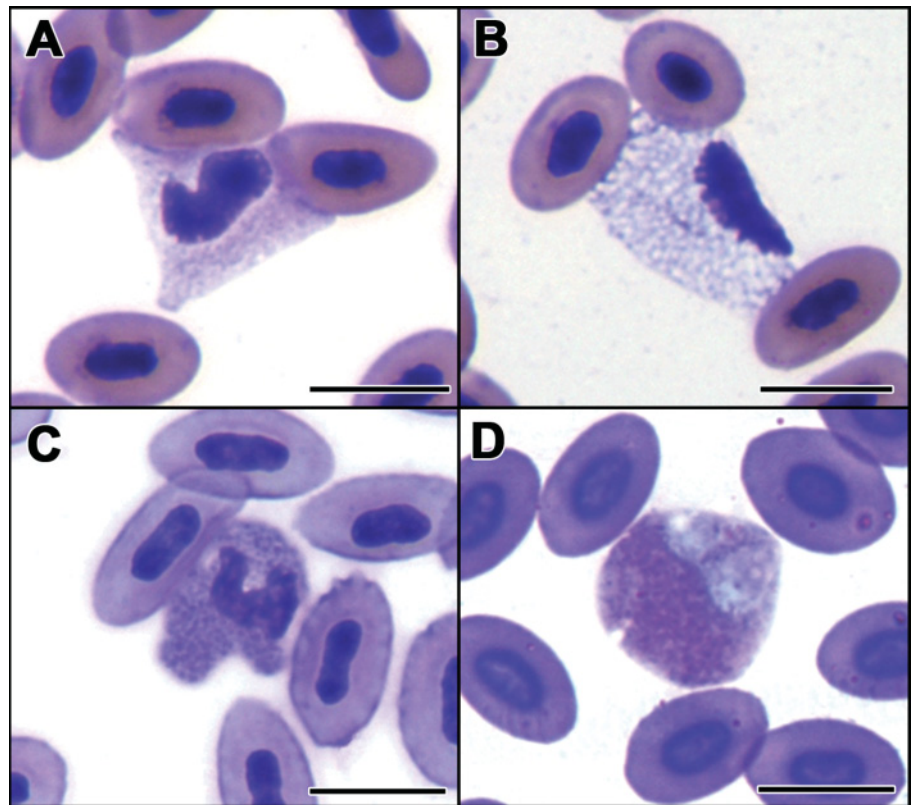


FIGURE 126.5 Granulocytes. Other granulocytes. Scale bar, 10 μm . (A) Goldfish – *Carassius auratus*, with heparin; Hema 3 stain. (B) Goldfish – *Carassius auratus*, with heparin; Hema 3 stain. (C) Grass carp – *Ctenopharyngodon idella*, with EDTA; Hema 3 stain. (D) Pacu – *Colossoma macropomum*, with EDTA; Wright-Giemsa stain.



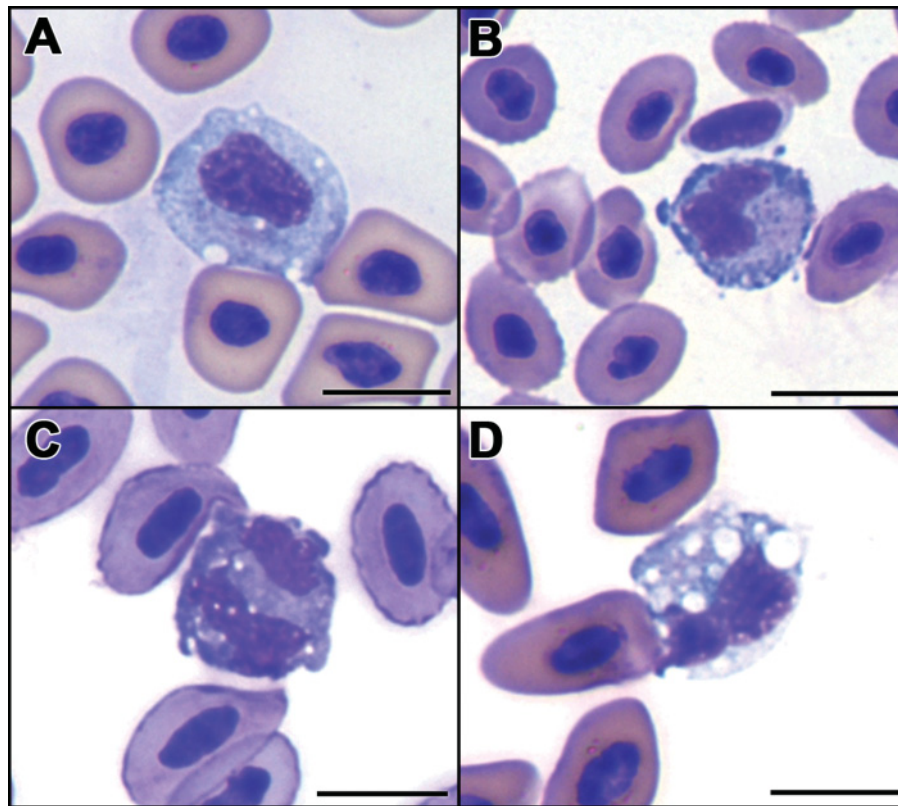


FIGURE 126.6 Monocytes. Monocytes are large cells with abundant dark blue staining cytoplasm that is frequently vacuolated and a variable shaped nucleus. Scale bar, 10 μm . (A) Catfish – *Ictalurus punctatus*, with heparin; Hema 3 stain. (B) Cobia – *Rachycentron canadum*, direct smear, no anticoagulant; Hema 3 stain. (C) Grass carp – *Ctenopharyngodon idella*, with EDTA; Hema 3 stain. (D) Koi carp – *Cyprinus carpio*, with heparin; Hema 3 stain.

BLOOD VALUES IN FISHES

Previously, the main focus of piscine hematology has been to determine the effects of toxicants and altered environmental conditions on the blood analytes. Frequently only a few analytes would be measured and hematologic changes were not correlated with reference intervals or with documented pathology or disease. This lack of knowledge provided a limited database on which to base diagnostic hematology. As such, hematological changes due to stress have been fairly well characterized in fishes, but other responses are not as well documented. Current work has focused on specific responses to pathogens, correlating hematologic changes with specific tissue pathology, and standardizing reference intervals. This work has significantly advanced the field of fish hematology although much work remains, particularly to standardize the nomenclature and function of the various blood cells.

Hematologic values for a number of common fish species are listed in Table 126.1. In general, the hematocrit ranges from 20% to 45%, with higher PCV values seen in active species of fish. The hematocrit can increase artifactually from splenic contraction and from RBC swelling that occurs after the sample is collected.¹⁹ Pre-capture stress or inadequate anesthesia during blood

collection can exacerbate RBC swelling and significantly increase the PCV.

Hemoglobin concentrations are low compared to mammals, being 5–10 g/dL. As with the PCV, more active fishes generally have higher hemoglobin values. The MCV ranges from 150 to 350 fL. Active fish with higher oxygen demands tend to have smaller RBCs and consequently lower MCV. The MCH varies considerably between species (between 30 and 100 pg) due to differences in size of circulating RBCs. The MCHC ranges from 18% to 30%, being lower in fishes than in mammals due to the space-occupying nucleus. The developing RBC continues to increase in size and hemoglobin content with time, resulting in higher MCV, MCH, and MCHC for older cells. Additionally, there may be a variety of maturational stages present at any time resulting in a wide range for these indices.

Red blood cell counts are usually lower in fishes than in mammals and vary with the need for oxygen. Fish that have a large surface area to body mass ratio (ice fish and some larval fish) have no discernible RBCs and are able to absorb sufficient oxygen directly from the water into the plasma. Sedentary fish have values closer to $1 \times 10^6/\mu\text{L}$, whereas active pelagic fish have counts greater than $5 \times 10^6/\mu\text{L}$. Leukocyte numbers are variable but generally range from 30,000 to 100,000 cells/ μL .

TABLE 126.1 Comparison of Hematological Values Determined for Various Fish Species

Species	No.	PCV (%)	Hgb (g/dL)	MCV (fL)	MCH (pg)	MCHC (g/dL)	RBC ($\times 10^6/\mu\text{L}$)	Reference
<i>Cyprinus carpio</i> (koi) ^a	30	29.7–33.8	6.3–7.6	166–190	37.7–42.7	20.4–22.9	1.69–1.91	54
<i>M. saxatilis</i> \times <i>M. chrysops</i> ^a	50	23–47	8–12	81–106	19.6–26.4	22–30	3.66–4.96	22
<i>Oncorhynchus mykiss</i> ^a	200	24–43	5.3–9.5	—	—	21.9–24.1	—	56
<i>Oncorhynchus mykiss</i> ^a	122	21–44	1.5–7.7	192–420	14.4–70.0	5.6–24.4	0.77–1.67	39
Tilapia ^a	40	27–37	7.0–9.8	115–183	28.3–42.3	22–29	1.91–2.83	25
<i>Chanos chanos</i> ^a	283	22–48	5–15	133–302	20.9–47.2	11–38	1.70–4.00	44
<i>Morone saxatilis</i>	18	34–55	6.2–10.9	155	31.3	20.5	2.0–4.2	36
<i>Carassius auratus</i>	60	38–40	9.7–10.6	241–245	63–66	26	1.6–1.8	5
<i>Ictalurus punctatus</i>	35	40	—	—	—	—	2.44	16
<i>Salmo salar</i>	20	44–49	8.9–10.4	441–553	94–106	19.4–21.7	0.85–1.1	48
<i>Salvelinus fontinalis</i>	74	36.6	8.0	—	—	—	1.34	20
<i>Oncorhynchus aquabonita</i>	12	44–52	—	—	—	—	1.19–1.20	29
<i>Sarotherodon melanotheron</i>	40	31–34	7.3–9.0	203–228	54	24–26	1.37–1.69	35
<i>Pleuronectes americanus</i>	~20	17–26	4.2–6.0	90–126	25–33	—	1.7–2.6	4
<i>Pleuronectes americanus</i>	~20	21–28	2.8–6.4	101–126	15–26	—	1.8–2.5	38
<i>Colossoma brachypomum</i>	29	25	—	—	—	—	1.68	53

Species	No.	WBC (/μL)	Lymphocytes (/μL)	Neutrophils/Heterophils (/μL)	Monocytes (/μL)	Eosinophils (/μL)	Thrombocytes (/μL)	Reference
<i>Cyprinus carpio</i> (koi) ^a	30	19,900–28,100	14,700–23,500	1,570–3,900	460–960	—	—	54
<i>M. saxatilis</i> \times <i>M. chrysops</i> ^a	50	32,600–115,100	22,500–115,100	400–3,500	1,500–7,500	0–400	30,700–74,100	22
Tilapia ^a	40	21,600–154,700	6,800–136,400	600–9,900	400–4,300	35–1,600	25,100–85,200	25
<i>Chanos chanos</i> ^a	195	17,500–92,500	51–68%	3–7%	4–9%	—	12–32%	44
<i>Crassius auratus</i>	55	10,100–14,700	9,540–13,660	—	—	—	30,000–46,100	41
<i>Ictalurus punctatus</i>	40	27,460–41,523	5,380–11,581	2,949–7,459	12,529–22,748	—	58,802–99,569	52
<i>Ictalurus punctatus</i>	35	164,000	89,900	5,200	500	0	68,400	16
<i>Oncorhynchus aquabonita</i>	5	21,000	18,799	1,582	588	—	135,000–310,000	29
<i>Sarotherodon melanotheron</i>	40	61,900–62,900	10.9–11.6%	3.1–3.8%	3.6–4.2%	—	80.5–82.3%	35
<i>Pleuronectes americanus</i>	~20	88,000–282,000	38,700–154,540	2,470–26,630	—	—	36,480–115,500	4
<i>Colossoma brachypomum</i>	22	33,500	21,028	3,183	1,242	209	—	53

^aDenotes that the range given is a reference interval; other values or ranges were determined for mixed groups of fish with various blood collection methods, time of the year, ages, sex, etc.

In most species, lymphocytes are the most abundant leukocyte present, followed by monocytes or neutrophils and eosinophils. Basophils, when present, are only occasionally seen.

In fishes, stress causes a leukopenia characterized by a lymphopenia and a neutrophilia.^{7,47} Fishes also demonstrate a typical inflammatory response consisting of a neutrophilia and lymphocytosis and increased serum protein concentration.^{45,49} Alterations in blood values have been noted with seasonal variation,^{34,38} water temperature,^{12,23} extreme pH,⁸ age, and sex,^{14,26} and stocking density.^{22,40} Observed seasonal changes might be associated with reproductive status (i.e. spawning cycle) and not seasonal changes per se.

A few studies have begun to document alterations in hematological profiles from specific diseases. Chinook salmon infected with bacterial kidney disease increased leukocyte counts to levels above controls after 16 days.³⁰ Rainbow trout infected with viral hemorrhagic septicemia had a decrease in PCV, RBCs, and hemoglobin.⁴⁶ Carp infected with *Aeromonas hydrophila* had increased WBCs and decreased RBCs, PCV, and hemoglobin. Values remained altered for more than 30 days after resolution of the infection.¹⁸ The decrease in RBC count is fairly consistent between studies, but it is unclear if it is due to altered red cell numbers or due to a change in hydration status.

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Hematology of Reptiles

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Sample Collection
 Sample Processing
 Blood Cell Enumeration
 Reference Intervals
 Erythrocytes
 Thrombocytes

Leukocytes
 Heterophils
 Eosinophils
 Basophils
 Lymphocytes
 Monocytes and Azurophils
 Hematopoiesis
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Acronyms and Abbreviations

EDTA, ethylenediaminetetraacetic acid; Hgb, hemoglobin; MCV, mean corpuscular volume; PCV, packed cell volume; RBC, red blood cell; WBC, white blood cell.

Reptiles have fascinated scientists for many centuries. Some of the earliest published reptilian hematology studies in English and French language literature date to the early 1800s (reviewed by Pienaar in 1962).³⁶ Studies published in the past several decades have investigated the ultrastructure, cytochemistry, function, physiologic responses, and other aspects of reptilian blood cells. Interest in reptilian hematology has probably increased together with the popularity of reptiles as pets, the willingness of clients to bring their reptilian pets to veterinarians, and with increased awareness of biodiversity and conservation of reptiles.

Evaluation of blood smears and hemogram data from reptiles can be difficult for veterinarians, veterinary technicians, and other animal biologists. This difficulty arises from differences in morphology of reptilian blood cells from those of mammals, differences in the morphology of leukocytes between reptilian species, lack of robust automated cell enumeration technologies that reliably work with reptilian blood, variation in study methods, and a less robust understanding of reptilian blood physiology and hematology when compared to mammals. Therefore, proper evaluation of reptilian hemograms and blood smears requires appropriate training, experience, equipment, and laboratory methods. This chapter will briefly review different aspects of reptilian hematology. The reader is referred to the list of references at the end of the chapter for

additional information, particularly some excellent reviews.^{3,9,39,41,42,47,48,49}

SAMPLE COLLECTION

Obtaining blood samples from reptiles for hematologic evaluation is readily accomplished with proper knowledge of anatomy, adequate training, and practice. Blood sampling techniques, such as toenail clips, tail tip clips, and decapitation, as used in some published studies, should be avoided and should not be a substitute for proper venipuncture. Comprehensive descriptions of venipuncture techniques are beyond the scope of this chapter and are well described in the literature.^{3,41,47,48}

SAMPLE PROCESSING

Detailed reviews of processing and handling of samples are reviewed elsewhere.^{3,39,41,48} Despite the small volumes that are obtained from some small patients, enough blood should be available to measure total plasma solids (using a heparinized capillary tube), packed cell volume (PCV), and blood smear evaluation. Blood smears prepared from fresh, non-anticoagulated whole blood are preferred.³ Ethylenediaminetetraacetic acid (EDTA) or lithium heparin may be used as

anticoagulant; however, EDTA may induce in vitro hemolysis of red blood cells (RBCs) in some species of reptiles, such as turtles and tortoises, and heparin anticoagulant may give a blue tinge to blood smears and induce clumping of leukocytes and thrombocytes.^{3,22} Lithium heparin should be used as an anticoagulant when the same blood sample will be used for biochemical analyses because potassium or sodium heparin may interfere with determination of electrolytes.

There are several types of staining methods that can be used to stain blood smears from reptiles. Diff Quik® and similar “quick” stains are commonly used in veterinary clinical practices. Literature descriptions of blood cells were based on a variety of staining methods including quick-type stains, Wright’s, Giemsa, Wright-Giemsa, Wright-Leishman, and others.

BLOOD CELL ENUMERATION

Cell enumeration in many species of mammals is routinely achieved with the use of automated cell counters.²⁸ These counters have been used to enumerate reptilian RBCs.^{21,24,31,39,44} Unfortunately, RBCs and thrombocytes are nucleated and this interferes with the capacity of the automated cell counters to separate reptilian blood cell populations.³ Automated cell counting technology probably can be used routinely for analysis of reptilian samples but this will involve additional research and development.

Because of the difficulties associated with enumerating reptilian blood cells in automated cell counters, the cell enumeration technologies available to practitioners and veterinary clinical pathologists have not changed significantly in many years. Hemacytometer-based techniques are routinely used to enumerate RBCs and white blood cells (WBCs) using solutions such as Natt and Herrick’s.^{3,39,48} These have inherent variability and limitations. The Unopette systems for RBC or eosinophil enumeration (Beckton Dickinson) were commonly used, but are no longer readily available. Estimation of WBCs or thrombocytes using a blood smear is another method that can be utilized but also has limitations.^{8,21,22,50}

REFERENCE INTERVALS

Reference intervals for many species of reptiles are available but should be interpreted with care because reference intervals published by one source may vary from those published by other sources in regards to season, geographical location, collection methods, venipuncture site, captivity status, restraint methods, analytical methodology, age and/or other factors.

ERYTHROCYTES

The PCV, RBC count, and hemoglobin (Hgb) of individual animals will vary with many factors including season, ambient temperature, sex, nutritional status,

and other factors.^{9,49} In general, reptiles have lower numbers of circulating RBCs than birds or mammals and there is an inverse relationship between the size of the RBC and the total number of circulating RBCs.⁴⁹ Additionally, reptiles with lower mean corpuscular volume (MCV), such as lizards, tend to have higher numbers of RBCs than others such as turtles.^{9,49} The average lifespan for reptilian RBCs (600–800 days) is typically greater than that of mammals and this may be due to a slower metabolic rate.⁴⁹

Reptilian RBCs are ellipsoidal with centrally positioned nuclei (Figs. 127.1 and 127.3). The cytoplasm is orange-pink and homogeneous in appearance with most stains. The nuclear chromatin becomes denser and darker as the cell matures. Young reptiles, those undergoing ecdysis or with regenerative anemia will frequently have increased numbers of immature RBCs in blood.³ Immature RBCs are typically less oval, have increased cytoplasmic basophilia, and have nuclei that also are less oval (Fig. 127.1). Similar to mammals, increased polychromasia is an indicator of erythroid regeneration secondary to decreases in the erythroid mass. Reticulocyte counts can be performed; however, there is very little published information on reticulocyte counts in reptiles.⁴³ Reptiles have punctate and aggregate reticulocytes.⁴³ Mitoses, binucleation, and other nuclear findings occur in reptiles with erythroid regenerative responses, inflammatory disease, post-hibernation, post-surgery, and erythroid neoplasia (Fig. 127.1).^{3,16} Likewise, minimal amounts of anisocytosis, polychromasia, and poikilocytosis occur in clinically normal reptiles but may be exaggerated in disease.

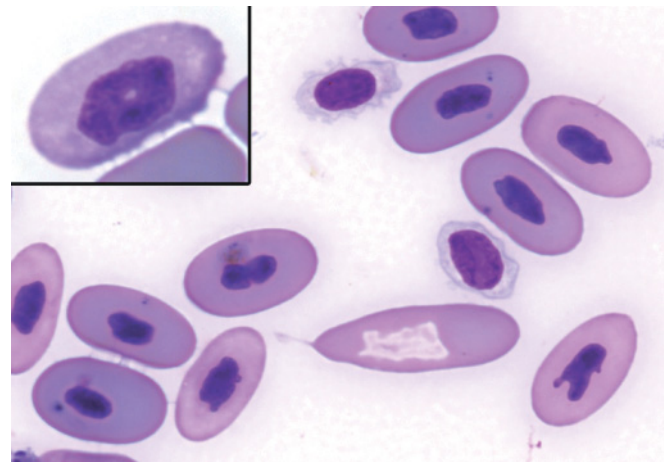


FIGURE 127.1 Blood smear from an inland bearded dragon (*Pogona vitticeps*) showing poikilocytosis. Red blood cells with irregular nuclear membranes (in particular the bottom right of the field), two thrombocytes (right and top of the field), and a tear-drop RBC lacking a nucleus (bottom right) are shown. The inset is a greater magnification of an immature RBC with a ring-shaped nucleus from the same blood smear. This slide was overstained using a quick stain. (From Reagan W, Irizarry Rovira A, DeNicola D. Veterinary Hematology: Atlas of Common Domestic and Non-Domestic Species. Ames: Wiley-Blackwell. 2008.)

Erythrophages occur with surgery and in other disease conditions.¹⁶ Parasites, including viruses, may infect RBCs.^{3,51} Single, small, round, basophilic inclusions in RBCs are found in some species of reptiles and have no pathologic significance.^{3,58} These have been confused with infectious agents.

THROMBOCYTES

Reptilian thrombocytes commonly aggregate in blood smears and share some functional characteristics with mammalian platelets.³ Thrombocytes are ellipsoidal to fusiform in shape with a centrally placed round to oval, darkly staining nucleus (Fig. 127.1). The cytoplasm is colorless to very pale blue and occasionally may contain azurophilic granules.³ When activated, the cytoplasm may show vacuolization, and develop a spherical shape.

Information on the thrombocyte count in the blood of reptiles is limited when compared to other reptilian blood cells.⁴⁹

LEUKOCYTES

Reptilian leukocytes frequently present a diagnostic dilemma because morphology varies, at times significantly, between reptilian species. This can lead to misclassification of reptilian cells. For example, the eosinophils of the green sea turtle (*Chelonia mydas*) have two morphologically distinct eosinophils,⁵⁸ and have been morphologically misclassified as neutrophils.¹ Reptilian leukocytes are typically classified as heterophils, lymphocytes, eosinophils, basophils, and monocytes/azurophils (Fig. 127.2). This terminology will be used here.

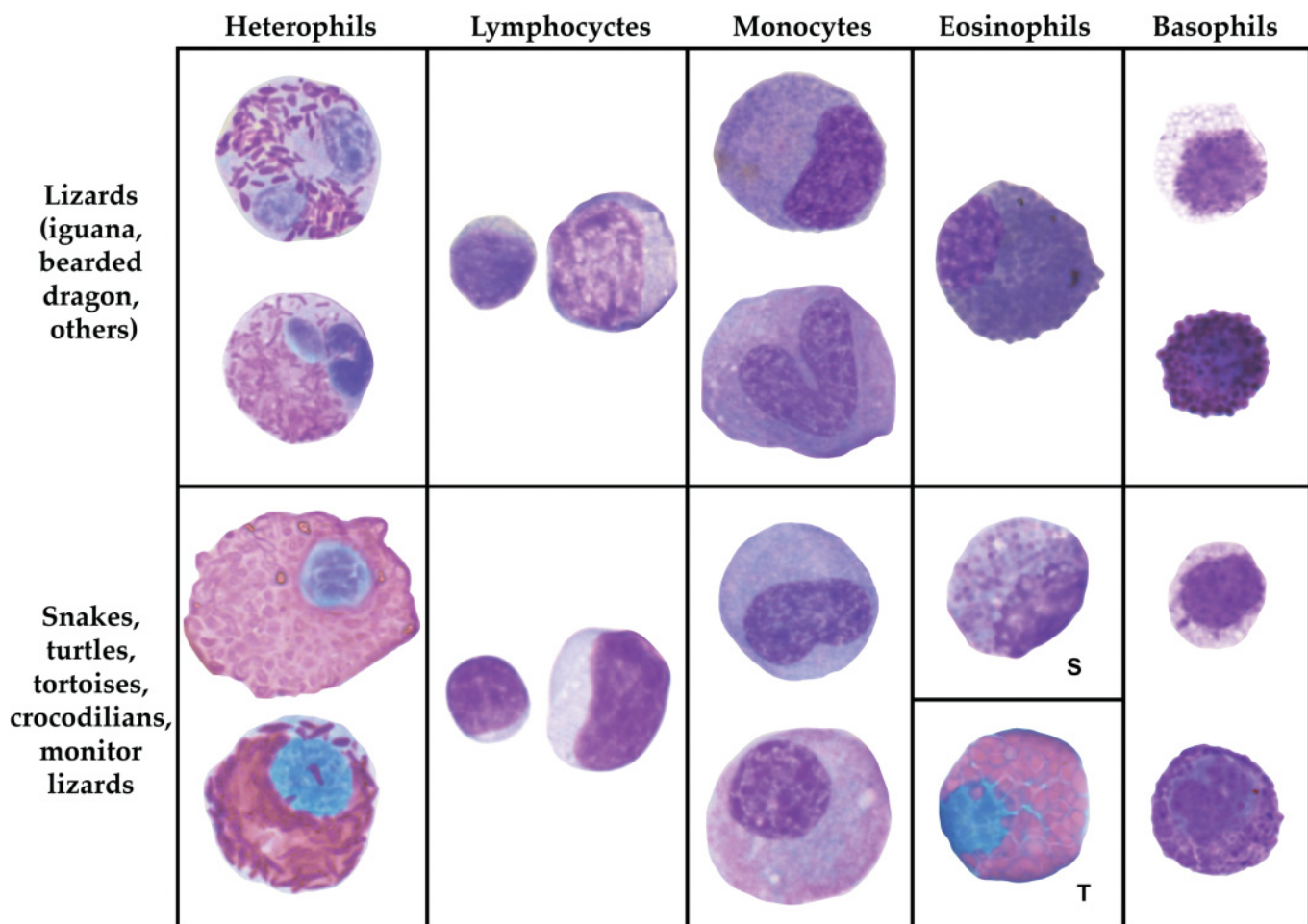


FIGURE 127.2 Overview diagram of leukocytes in healthy reptiles. S = snake; T = turtle (Adapted from Reagan W, Irizarry Rovira A, DeNicola D. Veterinary Hematology: Atlas of Common Domestic and Non-Domestic Species. Ames: Wiley-Blackwell. 2008.)

Heterophils

Functionally, the reptilian heterophil is considered analogous to mammalian neutrophils.³ Together with monocytes, they respond to tissue inflammation quickly, and have an important function is phagocytosis.^{9,32,49,54} Increases in numbers of blood heterophils may occur with inflammation and stress, and numbers also vary with season, and other factors.^{3,9} Coloration and morphologic variation in granules exist between reptilian species, but, in general, granules will be spindle-shaped to elongate and have an orange-red or “muddy brown” color.^{3,49} Heterophil granules in blood smears stained with “quick” stains may be less distinct than those stained with other methods such as modified Wright’s (Fig. 127.3).³ Crocodylians tend to have larger granules, although these are fewer in number than the numerous, smaller granules seen in lizards and snakes. The granules in snakes and turtles may be so numerous that granule shape will be difficult to discern. In general, the nucleus of heterophils from turtles, crocodylians, snakes, and some lizards (such as the monitor lizard, *Varanus* sp.) is round to oval while the nucleus of other lizard species, such as the green iguana (*Iguana iguana*) and the inland bearded dragon (*Pogona vitticeps*), is lobed.⁹ Similar to mammalian neutrophils, reptilian heterophils exhibit morphologic changes during inflammation including left shifts and toxic changes (Fig. 127.3).

Eosinophils

Reptilian eosinophils are round cells with spherical cytoplasmic granules and round to lobed nuclei.^{3,49} The color of the granules, shape of the nucleus, and size of the cells will vary with species. In many species of reptiles the granules of the eosinophil are bright red to orange; however, the granules of some reptilian species, such as the green iguana (*Iguana iguana*) and the inland bearded dragon (*Pogona vitticeps*), will have a blue to gray hue with Romanowsky-type stains (Fig. 127.2). Eosinophil numbers vary in relationship to multiple factors, such as species, season, and parasitic infestation.³

Basophils

Basophils have dark purple staining spherical granules with a slightly eccentric to centrally placed round to oval nucleus (Fig. 127.2). The nucleus is frequently obscured by darkly stained purple granules.³ Basophils may appear vacuolated with some staining methods (Fig. 127.2). Reptilian basophils probably function in a manner similar to mammalian basophils.³ The numbers of basophils vary with species, parasitic infestation, and other factors. In general, turtles have greater proportions of basophils than other reptilian species; however, other reptiles, such as newborn *Bothrops jararaca* snakes and some skinks, also have notable numbers of circulating basophils.^{6,59}

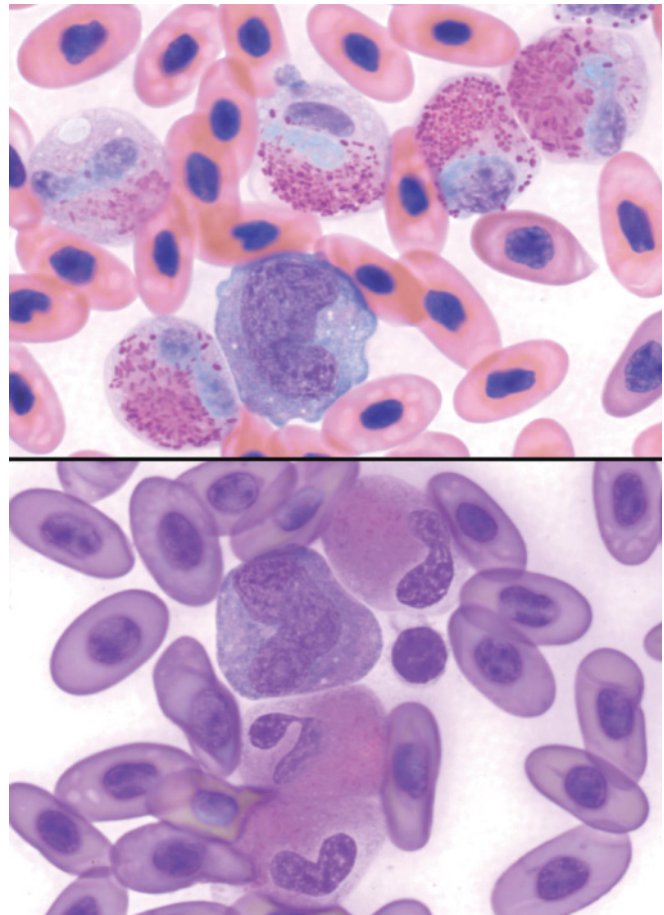


FIGURE 127.3 Blood smear from a green iguana (*Iguana iguana*). Heterophil with left shift and toxic change. Upper image: The five round cells with lobulated to oval nuclei, red round to oval granules in cytoplasm, and sometimes vacuolated are immature (i.e. left-shifted) and toxic heterophils. A large monocyte is in the bottom center of the field. Lower image: Three left shifted heterophils (top and bottom center of the field), a monocyte, and a small lymphocyte are shown. The lower image was from a blood smear stained with a quick stain; the blood smear shown in the upper image was stained with Wright’s stain. (From Reagan W, Irizarry Rovira A, DeNicola D. *Veterinary Hematology: Atlas of Common Domestic and Non-Domestic Species*. Ames: Wiley-Blackwell. 2008.)

Lymphocytes

The reptilian lymphocyte is morphologically similar to mammalian lymphocytes (Figs. 127.2 and 127.4). They are round with small amounts of basophilic cytoplasm and rarely contain azurophilic granules. Reptiles typically have small and large lymphocytes. Small lymphocytes may be confused with thrombocytes. The percentage of blood lymphocytes varies with season and other factors.^{9,49} For many reptiles, lymphocytes are the most prevalent circulating cell.⁴⁹ Lymphocytosis can occur with inflammation, wound healing, viral diseases, and certain parasitic infestations.³ Reactive lymphocytes and plasma cells rarely occur in blood and are

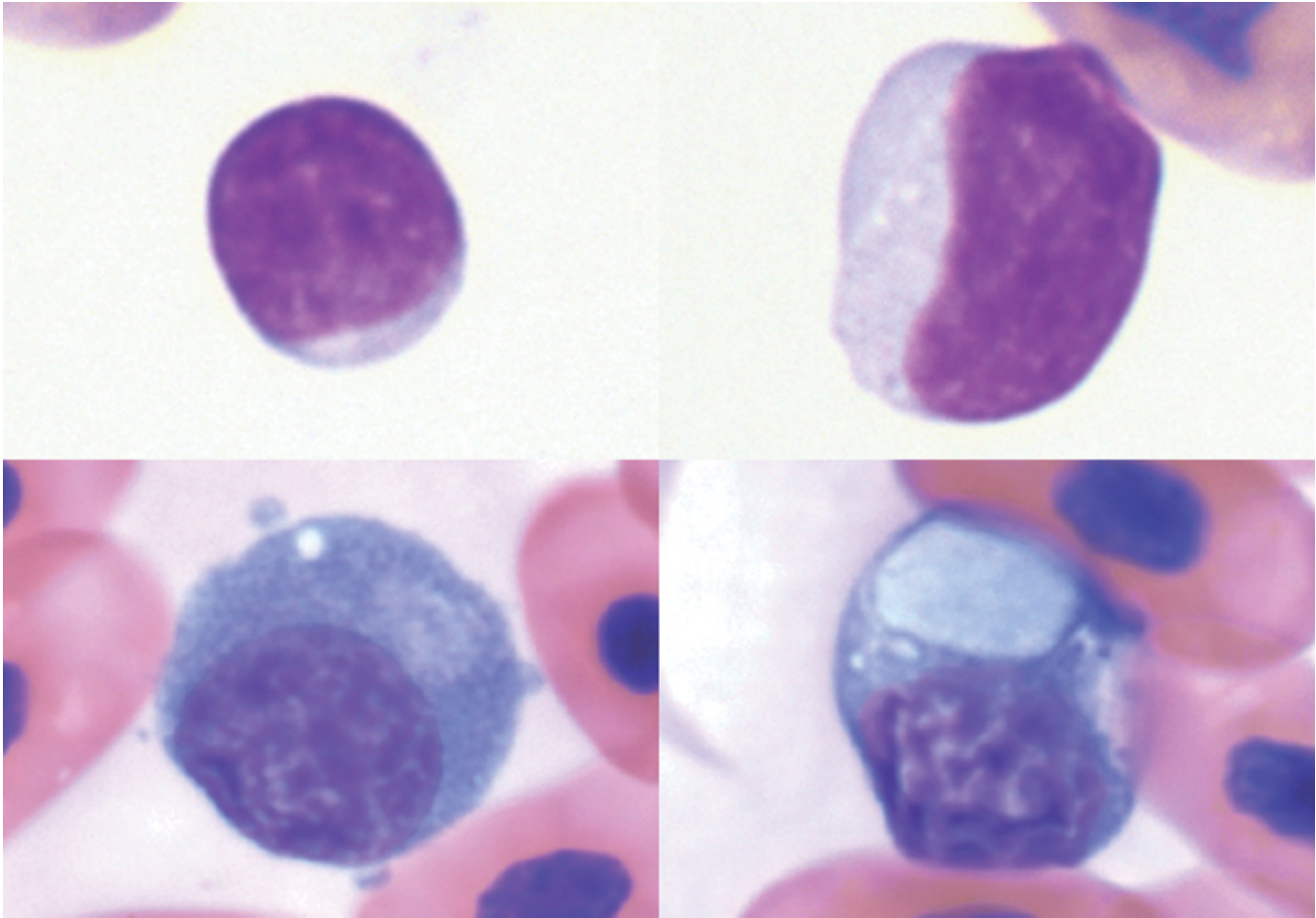


FIGURE 127.4 Representative images of lymphocytes from reptiles. The top two images are small (left) and large (right) lymphocytes from a snake (unspecified genus), and the bottom two images are from a green iguana (*Iguana iguana*). The lymphocyte in the bottom left is a reactive plasmacytoid lymphocyte, and the cell on the bottom right is a plasma cell with Russell bodies. Wright's stain. (Adapted from Reagan W, Irizarry Rovira A, DeNicola D. *Veterinary Hematology: Atlas of Common Domestic and Non-Domestic Species*. Ames: Wiley-Blackwell. 2008.)

similar in morphology to their mammalian counterparts (Fig. 127.4).³

Monocytes and Azurophils

The reptilian monocyte is similar in appearance to its mammalian counterpart (Fig. 127.2). It has moderately blue-gray cytoplasm, with or without vacuoles, and nuclei that vary from round to oval to indented.³ However, monocytes with fine, dust-like pink (azurophilic) cytoplasmic granulation have been referred to as azurophils (Fig. 127.2). The cellular origin of this cell has been the subject of debate for a long time. Some investigators classify all azurophils as monocytes³ while others indicate that azurophils from species such as snakes should be classified separately.⁴⁷ The clinical significance of classifying the azurophil as a separate cell type is unclear and has not been investigated sufficiently. The author prefers to group azurophils with monocytes.

HEMATOPOIESIS

There are limited numbers of studies or reviews of reptilian bone marrow hematopoiesis.^{3-5,7,10,14,15,20,27,33,36,40,45,49,55-57,60} Other studies have investigated the morphology of immature RBCs in circulation or hematopoiesis in other tissues other than bone marrow. What is understood is that bone marrow is the main location for production of blood cells in adult reptiles, although extramedullary hematopoiesis may occur in other organs, such as the spleen and liver.^{3,15,47} Variable amounts of active hematopoietic tissue occurs in vertebrae, long bones, ribs, carapace and plastron of turtles and tortoises, and other sites.^{14,15,45} In general, the morphology of reptilian hematopoietic precursors is similar to their mammalian counterparts with obvious exceptions related to the known morphologic differences from mammalian blood cells described in the preceding section. Although bone marrow responses have not been studied as extensively as in

mammals, evaluation of reptilian bone marrow can be performed to assess hematopoietic activity in patients.^{14,41} However, some studies suggest that bone marrow aspiration may be of limited value in some species of reptiles, such as desert tortoises, due to the lack of gelatinous bone marrow. In these cases bone marrow core biopsy may be necessary for adequate evaluation.^{14,15} Clearly, additional investigations of hematopoietic responses and cytologic evaluation of bone marrow tissue in reptiles are needed.

CONCLUSION

The present review was intended as a brief introduction to reptilian hematology. Interested practitioners would greatly benefit from review of the current literature, training with experienced colleagues, and routine

review of blood smears from reptilian patients, particularly those most frequently seen in private veterinary practices. Offering in-house hematological evaluation (including cell enumeration and other assays) to clients would be ideal if performed correctly. Furthermore, published reference intervals should be used with caution because there are many factors that influence the hemogram of reptilian patients, and there is variation in handling and analytical methods used to determine cell numbers. Tables 127.1–127.2 are a very small sample of the reptilian hemogram data that is available in the literature.

ACKNOWLEDGMENT

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TABLE 127.1 Erythrocyte Parameters of Selected Reptiles

Species	PCV (%)	Hgb (g/dL)	RBC ($\times 10^6/\mu\text{L}$)	Reticulocytes (%)	Reference
	Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean	
<i>Bothrops jararaca</i>	23.3 \pm 2.6	7.3 \pm 1.6	0.43 \pm 0.08	—	6
<i>Bothrops leucurus</i>	23.4 \pm 7.1	7.0 \pm 2.6	0.46 \pm 0.11	—	19
<i>Caretta caretta</i>	33.1 \pm 2.4	8.6 \pm 0.8	0.28 \pm 0.03	—	37
<i>Chelonia mydas</i>	29.0 \pm 4.0	— ^b	—	—	58
<i>Corucia zebrata</i>	35.5 \pm 9.0	9.6 \pm 1.3	1.45 \pm 0.29	—	59
<i>Crocodylus niloticus</i>	17.9 \pm 2.0	7.1 \pm 1.0	0.59 \pm 0.12	—	30
<i>Crocodylus palustris</i>	25.5 \pm 2.5	8.6 \pm 0.8	0.80 \pm 0.12	—	46
<i>Crotalus durissus</i>	22.7 \pm 3.6	11.5 \pm 4.3	1.56 \pm 0.13	—	53
<i>Cyclura c. inornata</i>	29.1 \pm 3.7	—	—	—	25
<i>Dermochelys coriacea</i>	36.0 \pm 5.4	—	0.38 \pm 0.20	—	8
<i>Eunectes murinus</i>	24.1 \pm 4.8	—	—	—	2
<i>Gallotia bravoana</i>	39.0 \pm 3.0	8.2 \pm 0.5	1.36 \pm 0.17	—	23
<i>Geochelone gigantea</i>	17.0 \pm 5.0	5.4 \pm 1.5	0.40 \pm 0.10	—	44
<i>Gopherus agassizii</i>	26.6 \pm 1.4 ^a	7.3 \pm 0.4 ^a	0.61 \pm 0.04 ^a	—	18
<i>Heloderma horridum</i>	35.9 \pm 3.8	8.9 \pm 1.0	0.86 \pm 0.10	—	12
<i>Iguana iguana</i>	38.0 \pm 3.7	10.6 \pm 1.2	1.40 \pm 0.10	—	21
<i>Ophiophagus hannah</i>	32.7 \pm 4.8	10 \pm 1.4	1.00 \pm 0.13	2.3 A; 19.7 P ^c	43
<i>Podocnemis expansa</i>	25.1 \pm 6.9	6.5 \pm 1.2	0.28 \pm 0.07	—	35
<i>Pogona vitticeps</i>	27.0 \pm 7.0	—	—	—	11
<i>Psammodromus algirus</i>	33.0 \pm 1.8	8.4 \pm 0.5	1.70 \pm 0.10	—	38
<i>Pseudemys rubriventris</i>	26.0 \pm 7.0	—	—	—	24
<i>Python molurus</i>	42.0 \pm 3.2	—	—	—	22
<i>Python regius</i>	18.3 \pm 5.3 ^a	6.7 \pm 2.6 ^a	—	—	26
<i>Testudo hermanni</i>	26.7 \pm 4.4	—	0.74 \pm 0.16	—	34
<i>Testudo marginata</i>	23.0 \pm 2.0	6.2 \pm 0.7	0.56 \pm 0.11	—	29
<i>Testudo radiata</i>	31.0 \pm 7.4	6.7 \pm 1.5	0.51 \pm 0.12	—	31
<i>Tupinambis merianae</i>	22.0 \pm 3.6	10 \pm 2.5	0.94 \pm 0.13	—	52
<i>Varanus komodoensis</i>	33.0 ^a	11.0 ^a	—	—	17
<i>Varanus salvadorii</i>	38.4 \pm 3.2	—	1.50 \pm 0.06	—	13

^aSE or median.

^b—, Not measured or not specified.

^cA, aggregate; P, punctate.

TABLE 127.2 Table of Leukocyte Parameters of Selected Reptiles

Species	WBC	Heterophils	Lymphocytes	Monocytes	Azurophils	Eosinophils	Basophils	Reference
	($\times 10^3/\mu\text{L}$)	($\times 10^3/\mu\text{L}$)	($\times 10^3/\mu\text{L}$)	($\times 10^3/\mu\text{L}$)	($\times 10^3/\mu\text{L}$)	($\times 10^3/\mu\text{L}$)	($\times 10^3/\mu\text{L}$)	
	Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD	
<i>Bothrops jararaca</i>	15.8 \pm 7.6	3.0 \pm 2.0	5.7 \pm 3.4	— ^b	1.7 \pm 1.9	—	5.4 \pm 2.2	6
<i>Bothrops leucurus</i>	11.6 \pm 8.2	0.6 \pm 0.7	7.5 \pm 5.6	—	2.4 \pm 2.8	—	0.5 \pm 0.3	19
<i>Caretta caretta</i>	3.7 \pm 1.0	2.2 \pm 0.7	1.1 \pm 0.8	0.0 \pm 0.0	— ^b	0.4 \pm 0.2	0.0 \pm 0.0	37
<i>Chelonia mydas</i>	13.8 \pm 5.3	1.4 \pm 0.8	10.0 \pm 4.3	0.8 \pm 0.5	—	1.7 \pm 0.6	0 \pm 0	58
<i>Corucia zebrata</i>	12.4 \pm 4.4	4.4 \pm 1.6	2.7 \pm 1.7	0.1 \pm 0.2	2.8 \pm 1.5	0.6 \pm 0.9	1.9 \pm 1.3	59
<i>Crocodylus niloticus</i>	11.3 \pm 4.7	2.1 \pm 0.8	7.2 \pm 3.8	0.1 \pm 0.2	0.6 \pm 0.7	0.5 \pm 0.6	0.7 \pm 0.7	30
<i>Crocodylus palustris</i>	7.0 \pm 1.3	4.5 \pm 1.2	1.8 \pm 0.6	0.1 \pm 0.1	—	0.6 \pm 0.2	0.0	46
<i>Crotalus durissus</i>	11.4 \pm 3.5	0.7 \pm 0.2	6.4 \pm 1.4	0.6 \pm 0.2	2.1 \pm 0.8	1.5 \pm 0.3	0.2 \pm 0.1	53
<i>Cyclura c. inornata</i>	6.9 \pm 2.6	4.3 \pm 1.8	1.3 \pm 0.8	0.2 \pm 0.2	0.0 \pm 0.1	0.1 \pm 0.2	0.5 \pm 0.3	25
<i>Dermochelys coriacea</i>	4.5 \pm 1.6	2.4 \pm 1.2	1.6 \pm 0.9	0.2 \pm 0.2	—	0.1 \pm 0.1	—	8
<i>Eunectes murinus</i>	13.2 \pm 6.5	2.8 \pm 1.5	5.1 \pm 2.9	0.2 \pm 0.2	5.2 \pm 4.3	—	0.2 \pm 0.0	2
<i>Gallotia bravoana</i>	3.8 \pm 1.2	2.6 \pm 1.2	0.4 \pm 0.2	—	0.7 \pm 0.3	0.0	0.2 \pm 0.1	23
<i>Geochelone gigantea</i>	3.4 \pm 2.3	3.0 \pm 2.3	0.7 \pm 0.5	0.1 \pm 0.1	—	0.0 \pm 0.1	0.1 \pm 0.1	44
<i>Gopherus agassizii</i>	7.8 \pm 1.2 ^a	4.9 \pm 1.2 ^a	1.0 \pm 0.2 ^a	0.8 \pm 0.2 ^a	—	0.3 \pm 0.1 ^a	0.8 \pm 0.2 ^a	18
<i>Heloderma horridum</i>	4.6 \pm 1.7	2.6 \pm 1.0	0.7 \pm 0.3	0.0 \pm 0.0	0.9 \pm 0.7	0.3 \pm 1.2	0.0 \pm 0.1	12
<i>Iguana iguana</i>	14.8 \pm 6.0	3.2 \pm 2.1	9.9 \pm 4.7	1.2 \pm 0.9	—	0.1 \pm 0.2	0.5 \pm 0.4	21
<i>Ophiophagus hannah</i>	17.3 \pm 4.7	1.4 \pm 1.0	13.6 \pm 3.1	0.0 \pm 0.0	2.2 \pm 1.7	0.0 \pm 0.0	0.1 \pm 0.1	43
<i>Podocnemis expansa</i>	6.1 \pm 2.3	2.9 \pm 1.1	0.9 \pm 0.3	—	0.4 \pm 0.2	1.4 \pm 0.7	0.5 \pm 0.3	35
<i>Pogona vitticeps</i>	12.1 \pm 4.1	3.3 \pm 1.6	7.1 \pm 2.4	0.1 \pm 0.2	0.4 \pm 0.3	—	1.1 \pm 0.7	11
<i>Psammmodromus algirus</i>	27.9 \pm 2.0	3.7 \pm 0.7	22.3 \pm 2.0	0.0 \pm 0.0	2.0 \pm 0.2	0.4 \pm 0.1	0.0 \pm 0.0	38
<i>Pseudemys rubriventris</i>	12.9 \pm 5.3	4.9 \pm 0.8	3.0 \pm 2.2	0.1 \pm 0.1	—	0.6 \pm 0.3	4.3 \pm 1.6	24
<i>Python molurus</i>	18.5 \pm 5.6	13.0 \pm 4.5	5.0 \pm 1.6	0.4 \pm 0.4	—	0.0	0.1 \pm 0.1	22
<i>Python regius</i>	12.2 \pm 2.0 ^a	6.8 \pm 4.5 ^a	1.7 \pm 3.3 ^a	0.1 \pm 0.1 ^a	1.6 \pm 1.2 ^a	—	0.1 \pm 0.2 ^a	26
<i>Testudo hermanni</i>	8.0 \pm 1.6	2.8 \pm 0.7	4.8 \pm 1.6	0.0 \pm 0.0	0.0 \pm 0.1	0.0 \pm 0.0	0.4 \pm 0.2	34
<i>Testudo marginata</i>	4.3 \pm 1.8	1.5 \pm 1.1	2.3 \pm 1.6	0.1 \pm 0.1	—	0.4 \pm 0.3	—	29
<i>Testudo radiata</i>	4.3 \pm 1.0	2.0 \pm 0.1	1.6 \pm 0.1	0.2 \pm 0.0	—	0.2 \pm 0.0	0.3 \pm 0.0	31
<i>Tupinambis merianae</i>	18.4 \pm 2.4	2.4 \pm 0.5	8.1 \pm 0.4	0.1 \pm 0.1	2.0 \pm 0.5	4.4 \pm 0.6	0.40 \pm 0.0	52
<i>Varanus komodoensis</i>	6.0 ^a	2.7 ^a	3.2 ^a	0.2 ^a	0.1 ^a	0.0 ^a	0.0 ^a	17
<i>Varanus salvadorii</i>	11.5 \pm 2.8	5.8 \pm 2.3	1.6 \pm 0.2	0.2 \pm 0.1	3.9 \pm 0.8	0.0 \pm 0.0	0.2 \pm 0.1	13

^aSE or median.^b—, Not measured or not specified.

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1012 SECTION IX: SPECIES SPECIFIC HEMATOLOGY

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Hematology of Elasmobranchs

MICHAEL K. STOSKOPF

Morphologic Characterization
Protein Concentration

Response to Infectious Disease
Response to Noninfectious Disease

Acronyms and Abbreviations

ATPase, adenosine triphosphatase; Hgb, hemoglobin; PAS, periodic acid-Schiff; PCV, packed cell volume; RBC, red blood cell.

The divergence of the elasmobranchs over their long evolution has resulted in over 500 species known to exist today. These species are adapted to many different ecologic niches, so variation in baseline hematologic parameters among the species of cartilaginous fishes should be expected. Unfortunately, the blood constituents of few elasmobranch species have been carefully investigated. Even less work has been published associating changes in hematologic parameters of elasmobranchs to the presence of disease. Recent work by Arnold¹ has provided guidance in standardizing hematological methods for use with elasmobranch blood samples. These methods will hopefully improve the quality of material being published on these species.

MORPHOLOGICAL CHARACTERIZATION

Elasmobranchs have nucleated red blood cells (RBCs) and circulating blast cells. Mitotic figures are frequently observed and are thought to be in the RBC series, although detailed studies to confirm this are not available (Table 128.1). All principal leukocyte types are found in elasmobranch blood^{1,4,15} (Table 128.2). Detailed electron microscopy and some cytochemistry studies of these cells are available.^{2,3,7,8,9,10,14} Morphological evaluation of elasmobranch blood cells can be readily accomplished using routine methods. The classification of cells into heterophils, (sometimes referred to as neutrophils; Figs. 128.1 and 128.2), eosinophils (Fig. 128.3), monocytes (Fig. 128.4), lymphocytes (Figs. 128.5 and 128.6) and thrombocytes (Fig. 128.6) on the basis of cytoplasmic granule morphology and staining intensity

in modified Wright-Giemsa stained smears is relatively straightforward. However, immature cells in the erythroid series are frequently erroneously counted as leukocytes. This is probably the basis for some reports of extremely high leukocyte counts with a high proportion of lymphocytes in various shark species.²⁴

A major controversy remains to be resolved regarding the nature of the predominant granulocyte cell types found in elasmobranchs. Despite efforts to classify the most frequently encountered granulocytes as eosinophils, all functional evidence points to their being true heterophils with activities much like the neutrophil of mammals.¹³ The question is complicated because both eosinophils and heterophils of elasmobranchs that have been studied are acid phosphatase and periodic acid-Schiff (PAS) positive.^{4,7} Grimaldi found heterophilic granulocytes to be more intensely PAS and aliesterase positive and weakly ATPase positive compared to eosinophilic granulocytes that show the presence of neutral polysaccharides in the matrix but have strongly ATPase and acid phosphatase positive granules.⁴ Some phagocytic activity has been demonstrated in elasmobranch heterophils.¹¹ A cell morphologically similar to granulocytes but without any cytoplasmic granules is frequently seen in blood of some species (M.K. Stoskopf, personal observation). It is not known if these represent a distinct cell type, are degranulated cells, or are cells that have not yet developed granules. More cytochemical studies are needed to elucidate the situation but, at present, such cells are routinely grouped with heterophils.

Elasmobranchs have efficient immunosurveillance and there is phyletic conservation of some cell mediator

TABLE 128.1 Baseline RBC Indices of Elasmobranchs

Species	N	Total RBC ($\times 10^6/\mu\text{L}$)	PCV (%)	Hemoglobin (g/dL)	Reference
Blue shark	14		22	6	12
Brown shark (captive)	20	0.532	20	<4	20
Bonnethead shark (wild)	21		24		6
Lemon shark (captive)	3	0.665	20	5	20
Nurse shark (wild)	5	0.366	10	4	20
Nurse shark (captive)	7	0.35	11	<4	20
Portuguese shark	1	—	13	—	19
Sandtiger shark (captive)	9	0.276	24	6 (8)	21
Spiny dogfish	21	—	19	5	22
Torpedo	15	0.201	25	23	16

TABLE 128.2 Baseline Leukocyte Indices of Elasmobranchs

Species	N	Total WBC ($\times 10^3/\mu\text{L}$)	Heterophils (%)	Bands (%)	Monocytes (%)	Lymphocytes (%)	Eosinophils (%)	Basophils (%)	Reference
Brown shark (captive)	20	28.1	58	0	1	40	1	0	21
Lemon shark (captive)	3	25.9							21
Nurse shark (wild)	5	27.8							21
Nurse shark (captive)	7	27.2	56	1.4	30	0	0		21
Nurse shark (unspecified) ^a	13		24	2.3	72	0	0		24
Sandtiger shark (wild)	8	16.1	45	1	19	28	0		22
Torpedo	15	43.8							17
Atlantic stingray (unspecified) ^a	10		29	1.6		69	0	0	24
Cleurnosed skate (unspecified) ^a	7	21	1.3			77	0	0	24

^aThese references report very high total WBC counts indicating that there is a high probability that erythroid cells were counted as lymphocytes resulting in the high lymphocyte percentage reported.



FIGURE 128.1 A heterophil (left) and an erythrocyte from a nurse shark (*Ginglymostoma cirratum*). Note the thin elongate cytoplasmic granules in the heterophil.

and regulatory products of lymphocytes and monocytes between elasmobranchs and humans.⁵ Elasmobranch lymphocytes, monocytes, and thrombocytes can show weak PAS staining. Lymphocytes may also have aliesterase positive granules, while monocytes occasionally show some small PAS positive granules and weak acid phosphatase and aliesterase activities.⁴ Phagocytosis has been demonstrated for elasmobranch cells considered to be monocytes but not for lymphocytes.¹¹ However, lymphocytes that appear to have engulfed material are seen in blood smears on occasion (Fig. 128.7).

Only a single thrombocyte cell type has been described in elasmobranchs. This is in contrast to similarly studied teleosts (*Rutilus rutilus* and *Gobio gobio*) that have two morphologic thrombocyte types.²⁵ The thrombocytes of elasmobranchs are rounded to spindle-shaped and originate in the spleen from prothrombocytes.¹⁷ They contain microtubules and cytoplasmic granules that seem to correspond functionally to the mammalian platelet canalicular system.^{17,25} The cytoplasmic presence of platelet factor 4, beta-thromboglobulin, and factor VIII-related antigen has been demonstrated by immunocytochemical staining. Elasmobranch thrombocytes adhere to glass, and aggregation and degranulation can be stimulated by



FIGURE 128.2 A young heterophil (near top) and a mature heterophil (below) among a group of mature erythrocytes, from a nurse shark (*Ginglymostoma cirratum*). The gradation in staining along the rubricytic series during maturation is more easily appreciated with a preliminary scan of the blood smear.

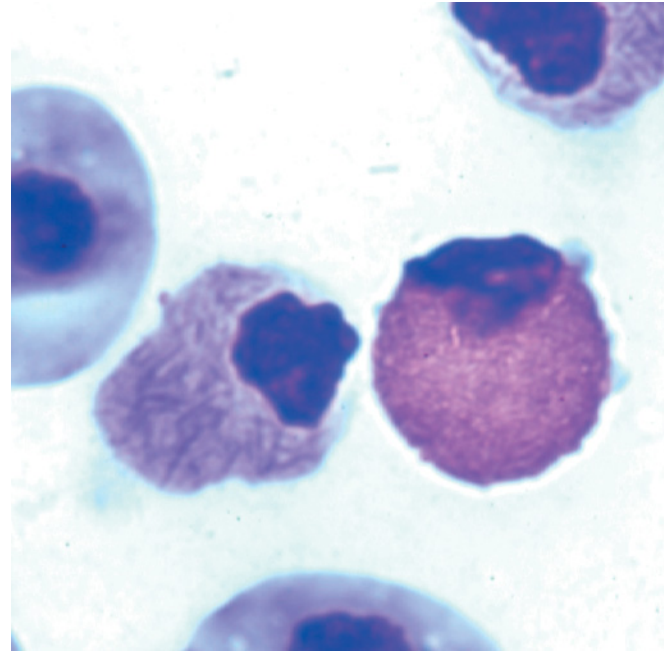


FIGURE 128.3 An eosinophil (right) and a heterophil (left) from a sandtiger shark (*Odontaspis taurus*) showing the eccentric nucleus and rounder more eosinophilic cytoplasmic granules of the eosinophil. Note the differences between the cytoplasmic granules in the heterophil compared to those in the nurse shark in Figure 128.1.

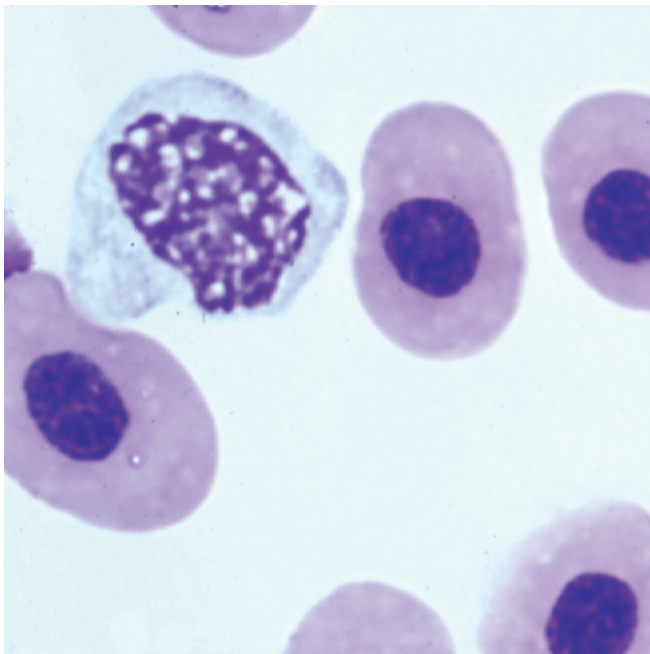


FIGURE 128.4 A monocyte from a brown shark (*Carcharhinus plumbeus*). Note the large nucleus and foamy cytoplasm of this leukocyte which is larger than found in young lymphocytes.

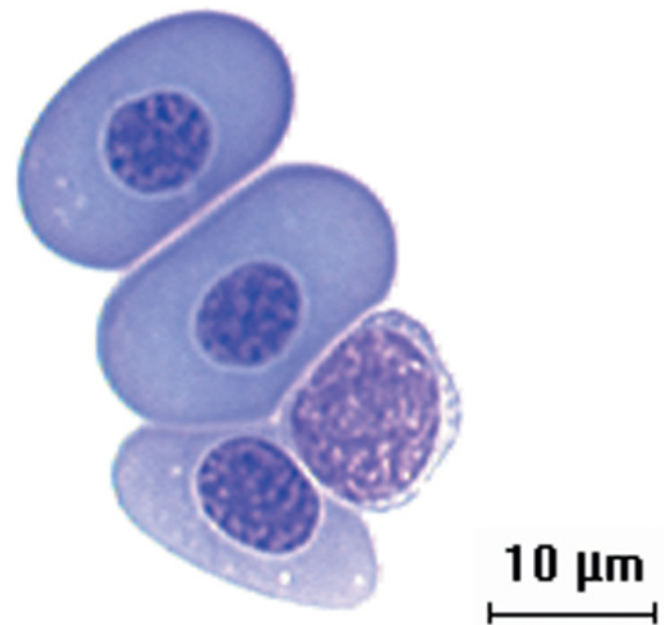


FIGURE 128.5 A large lymphocyte and two mature erythrocytes from a nurse shark, (*Ginglymostoma cirratum*).

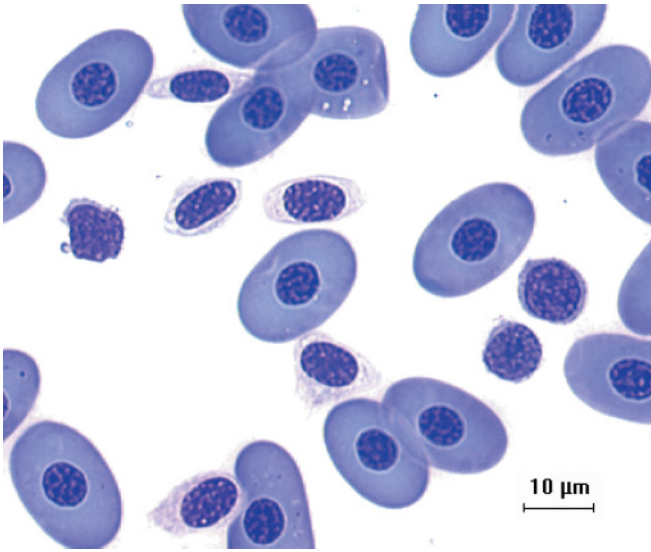


FIGURE 128.6 Five thrombocytes in various stages of maturity and three small lymphocytes from a nurse shark (*Ginglyomstoma cirratum*). Note the relatively elongate nucleus of the thrombocytes and the different staining quality of the cytoplasm of the two cell types.

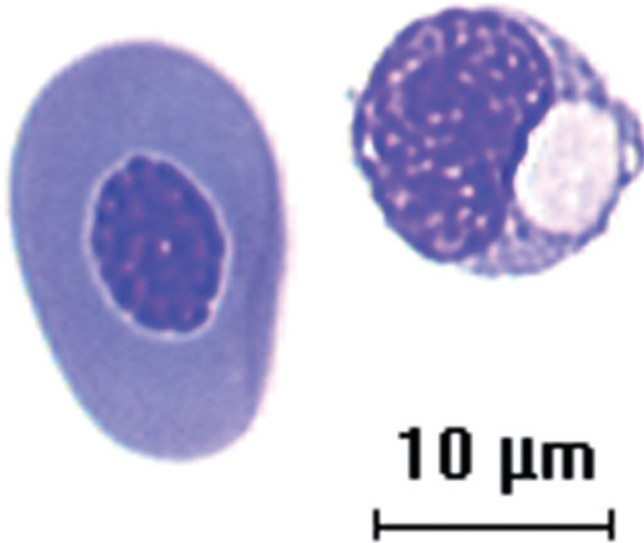


FIGURE 128.7 Lymphocyte from a nurse shark (*Ginglyomstoma cirratum*) with apparently phagocytized cellular debris.

collagen, noradrenaline, 5-hydroxytryptamine, thrombin, or adenosine diphosphate (ADP).¹⁷ Thrombocytes contain some peripheral granules that are PAS positive and slightly ATPase positive.⁴

PROTEIN CONCENTRATION

An important methodological problem in elasmobranch hematology and clinical pathology is the determination of serum or plasma protein. Determinations made by a

refractometer result in artificially high serum or plasma total protein concentrations, because of the urea nitrogen concentration that elasmobranchs maintain for the purposes of osmotic regulation. Refractometer total protein determinations are typically 2–3 times higher than automated colorimetric total protein assay results (Table 128.3).

RESPONSE TO INFECTIOUS DISEASE

Elasmobranch hematological parameters respond to infection with many of the same changes seen in mammals. Bacterial infections routinely cause marked leukocytosis, usually due to a heterophilia. A distinct left shift is difficult to demonstrate because mature granulocytes of elasmobranchs have less distinctly lobed nuclei than analogous cells of most mammals. This makes detection of immature cells less certain.

There has been little or no experience in detecting hematological changes associated with viral or protozoal diseases in elasmobranchs. Overwhelming trematode infection can be accompanied by a leukocytosis, but it is difficult to know whether this is a response to trematodes, a generalized stress response, or a response to secondary bacterial infections that can accompany heavy trematode infestations.

RESPONSE TO NONINFECTIOUS DISEASE

Stress is a frequent factor in interpretation of elasmobranch hemograms. Confinement stress appears to cause decreased red blood cell (RBC) counts, packed cell volume (PCV), and hemoglobin (Hgb) levels in spiny dogfish. In addition, a nonregenerative and, most likely, redistributional anemia develops, and serum glucose and total leukocyte count increase dramatically with confinement stress.²² This suggests that the stress leukogram of elasmobranchs may be difficult to differentiate from a response to bacterial septicemia. The leukocytosis in both instances is primarily due to a heterophilia, and an accompanying eosinopenia in the stress leukogram may not be easily appreciated.

Some elasmobranchs are found in freshwater environments and some species, such as the bull shark, move between fresh and saltwater environments as part of their natural history. These species appear to exhibit RBC swelling when exposed to acute reductions in environmental salinity beyond 50%. They respond by rapidly increasing serum urea nitrogen concentration and gradually shifting their electrolyte balances when moved acutely from freshwater to high salinity.¹⁸ It is reasonable to assume that environmental salinity should be considered when evaluating these parameters in elasmobranchs.

The other reasonably well documented noninfectious cause of hematologic changes in sharks, skates, and rays is heavy metal toxicity. Copper exposure is a frequent problem in captive animals and elasmobranchs are much more sensitive to this metal than marine

TABLE 128.3 Protein and Urea Nitrogen Concentrations of Elasmobranchs

Species	N	Urea Nitrogen (mg/dL)	Total Protein (g/dL)	Albumin (g/dL)	Globulin (g/dL)	Reference
Blacktip shark (captive)	2	1015	3.3	0.5	2.8	21
Brown shark (wild)	33	848 (34)	2.2	0.5	1.7	20
Bonnethead shark (wild)	24	1004	2.9	0.4	2.6	6
Dusky shark (wild)	5	912	1.7	0.6	1.1	20
Finetooth shark (captive)	3	983	2.4	0.4	2.0	21
Scalloped hammerhead shark (wild)	4	880	2.5	0.4	2.1	20
Lemon shark (captive)	2	1023	3.5	0.6	2.9	20
Nurse shark (wild)	9	1147	2.6	0.6	2.2	20
Nurse shark (captive)	12	1087	2.0	0.4	1.5	20
Sharpnose shark (wild)	10	847	2.1	0.4	1.7	20
Sandtiger shark (wild)	3	1027	3.3	0.6	2.7	21
Spinner shark (captive)	3	923	2.6	0.4	2.2	21
Tiger shark (captive, compromised)	2	1134	4.6	0.5	4.2	20

teleost fishes. A normochromic normocytic anemia with decreased total RBC count and PCV and a stable Hgb value has been documented in spiny dogfish exposed to sublethal concentrations of copper (2ppm for 48 hours). A leukopenia and decreased serum glucose was also seen.²³ Exposure to higher copper levels (4, 6, 8, or 16ppm) is fatal to spiny dogfish within 56 hours and causes similar anemia and falling Hgb concentrations.²³

Zinc exposure in spiny dogfish also causes a microcytic hypochromic anemia characterized by a decreased Hgb concentration, but the PCV generally remains stable while the total RBC count is increased. This toxicity is characterized by a leukocytosis in contrast to what is seen in copper exposure. Serum glucose is also decreased.²²

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SECTION X

Quality Control and Laboratory Techniques
Andreas Moritz

Quality Control

MADS KJELGAARD-HANSEN and ASGER LUNDORFF JENSEN

Quality Management

Quality Control

Quality Control Materials and Methods

Analytical Quality Specifications

Statistical Quality Control Rules

The Role of Proficiency Testing in Quality Control

The Role of Manual Slide Evaluation in Quality Control

White Blood Cell and Red Blood Cell Counts

Leukocyte Differential Count

Leukocyte Morphology

Reticulocytes

Red Blood Cell Morphology

Platelets

Acronyms and Abbreviations

CV, coefficient of variation; CV_G , inter-individual variation; CV_I , intra-individual variation; EQA, External Quality Assessment; ISO, International Organization for Standardization; QC, Quality Control; RBC, red blood cell; SOP, Standard Operating Procedure; WBC, white blood cell.

Quality control (QC) in some aspects resembles diagnostic test validation (Chapter 130) but there are significant differences. In general, laboratory measurements for clinical purposes are performed to answer a question of consequence to patient management. Evaluating whether a test is able to answer such questions appropriately is performed through test validation procedures when introducing the test to the laboratory. A main step in this process consists of answering the question, Will the test provide results with acceptable quality for clinical decision-making? To answer this question, several sources of variation are evaluated in the step-wise approach of the validation procedure, including analytical variation, biological-physiological variation, and pathological variation. It must be concluded that analytical and biological-physiological variation is of such a magnitude that pathological variation can be detected in a clinically useful manner. The allowable level of analytical and biological-physiological variation is thus determined in part by the magnitude of the pathological variation, as a marked shift in the level of the analyte in relation to disease will allow wider margins than if the shift is only subtle and therefore more easily masked by excessive analytical variation.

Following thorough validation procedure and a decision to apply the test in a clinical setting, the question of quality shifts to, Does the test *still* provide results

with acceptable quality for clinical decision-making? and this is where QC takes over from validation.

In this chapter, the major aspects of QC in veterinary hematology will be outlined. The ultimate task is to ensure that the test has an ongoing ability to detect pathological variation in a clinical useful manner. The focus will be on controlling analytical variation, as both biological-physiological variation and pathological variation are mainly patient-related and in a strict sense outside the scope of QC. However, knowledge of especially biological variation is of great benefit in the application of appropriate QC, as described later in this chapter.

QUALITY MANAGEMENT

The concept of total quality management will be described briefly to outline the role of QC within this system. Quality management has evolved through time to ensure that quality goals are fulfilled. Performing QC was added in medical laboratories in the 1960s to statistically monitor the performance of analytical processes.²⁰ Other aspects were added later to also include other quality characteristics, such as turnaround time and sample identification in the system, and in the 1990s a framework for continuous improvement of quality was applied.²⁰ This framework for quality management included the following: quality laboratory

practices (e.g. procedures and protocols), quality control, quality assurance, quality improvement, and quality planning.

Quality improvement and quality planning are tools to implement and plan changes outside the actual laboratory to correct or prevent quality problems, respectively.²⁰ In modern times the framework has been integrated into quality system standards, such as the International Organization for Standardization (ISO) 15189:2007 standard for medical laboratories.¹¹ The ISO 15189:2007 standard has been suggested as a blueprint for quality systems in veterinary laboratories.⁸

Quality management involves a holistic approach to address all identified aspects of quality, and to plan, control, intervene and improve in a continuous process. Quality control is that part of the process whereby analytical error is monitored statistically to detect errors of possible clinical importance. As a key component, it is addressed at several levels of the quality system standards.¹¹

To put QC in the context of the diagnostic process, the time from sampling to delivery of results can be split into three phases: the pre-analytical, analytical and post-analytical phase. Each phase has its own palette of sources of variation that should be kept at a level with no or negligible influence on the final clinical decision based on the reported result. Where QC is limited to the analytical phase and mainly based on a statistical approach, recommendations regarding the pre- and post-analytical phases are to apply rigorous Standard Operating Procedures (SOPs), taking important sources of variation into account. Important pre-analytical factors such as the amount of sample, use of anticoagulant, timing of sample, transport, handling and sample identification should all be accounted for in the SOP.¹⁰ For the post-analytical phase the key issue is to avoid “gross errors” that can have fatal consequence to patient management. Again appropriate SOPs are key tools, with the most important weapon against gross errors being to check and double check reports before releasing results or to allow application of smart Laboratory Information Systems.⁹

QUALITY CONTROL

Quality control itself consists of several parts that together enable the statistical control of random and systematic errors. These parts include: (1) quality control materials and methods, (2) analytical quality specifications, and (3) statistical quality control rules. All parts are necessary to perform appropriate QC, and the total efficacy of QC depends on thorough consideration in defining each part. One set of choices will not be optimal for every analyte in veterinary hematology and will not fit across veterinary laboratories, as demands on QC will vary with methodology and the use of the results. So each laboratory will have to choose in accordance with its own identified needs. It should be kept in mind that the total QC is not better than the

weakest part, e.g. if a lot of effort is put into defining appropriate quality specifications and application of QC rules, it is of little use if poor or unstable QC material is used. Likewise, the use of substantial resources to establish valid and stable control material is of little use if quality specifications and control rules are poorly defined.

Quality Control Materials and Methods

Regarding QC material, it should be noted that QC should be made at several levels of the analyte, optimally at least within the normal range, close to the clinical decision level and within the pathological range. When stable QC material is available, i.e. when the analyte is stable in patient samples or sample pools, or when stable control material is provided by manufacturer, this can then be applied according to defined QC rules, as described below. However, many analytes in veterinary hematology have sub-optimal stability to allow long term use of patient or patient-like material, and alternative QC methods must be used. Alternative methods may involve performing routine validation as described in detail in Chapter 130, e.g. routine method comparison between automatic (calculated) PCV and spun (measured) PCV can be performed to detect systematic error. The use of manual leukocyte differential count to evaluate automated differential count is another example of routine method comparison used for QC purpose to detect bias, where bias is defined as the difference between results of the actual measurement and the true value. If excess imprecision is suspected, as determined by the lack of close agreement between independent results on the same analyte, fresh patient samples at various levels can be run repetitively to assess random variation and compare to specified quality goals.

Analytical Quality Specifications

Quality control must be designed to reveal clinically important systematic and random error in order to identify and eliminate the source of variation. To do this, criteria to decide whether a level of error is important or not is mandatory, without which decisions of acceptability can only be arbitrary. Nevertheless, this requirement is seemingly tough to meet in veterinary laboratories, as it has recently been identified as one of the most frequent reasons for not meeting accreditation criteria.⁸ The generation of such quality specifications has also been subject to several discussions in human medicine and was the sole object of a meeting in Stockholm in 1999, resulting in a consensus statement on the setting of quality specifications in laboratory medicine.¹⁴ It was agreed that the following hierarchy of models should be applied to set analytical quality specifications:

1. Evaluation of the effect of analytical performance on clinical outcomes in specific clinical settings.

2. Evaluation of the effect of clinical performance on clinical decisions in general:
 - (a) Data based on components of biological variation
 - (b) Data based on analysis of clinician’s opinion.
3. Published professional recommendations
 - (a) From national and international expert bodies
 - (b) From expert local groups or individuals.
4. Performance goals set by
 - (a) Regulatory bodies
 - (b) Organizers of external quality assessment (EQA) schemes.
5. Goals based on the current state of the art
 - (a) As demonstrated by data from EQA or proficiency testing scheme
 - (b) As found in current publications on methodology.

It is important to make use of data ranking as high as possible in this hierarchy, as it will ensure optimal relevance of the finally established quality specification to the key question of QC, namely, Does the test provide results with acceptable quality for clinical decision-making? Only limited, if any, data at the level of “evaluation of the effect of analytical performance on clinical outcomes in specific clinical settings” are available in veterinary hematology. However, at the next level (2a), data on the biological variation of several analytes in dogs (Table 130.2, p 1028), as well as in other animal species¹² are available and should be used. A few other aspects of the ranking call for comments related to the veterinary application. There is no established tradition in veterinary medicine to define quality specifications based on the opinion of experienced clinicians, despite the high ranking of this method in the consensus statement. It has been suggested that highly relevant quality goals can be obtained by presenting well-defined case histories together with questionnaires to experienced clinicians.¹⁸ Clinicians are asked what changes in the concentration of an analyte will cause them to change actions concerning the patient, and the laboratory must be able to detect these changes with a certain probability. Interestingly, it should be noticed that “professional recommendations from expert local individuals” ranks higher in the consensus statement than quality specifications derived from External Quality Assessment (EQA), regulatory bodies and general publications on the methodology. The higher ranking is due to the knowledge of the local expert on local needs and specific settings of the methodology, which are not included in the lower ranking methods. So although this “expert opinion” is sometimes viewed as too subjective, this subjectivity is what makes it superior to the lower ranking, more general methods of defining quality specifications.

To further exemplify application of the consensus statement, the use of data on biological variation to define quality specifications will be outlined. Studying biological variation of an analyte results in estimates of intra- (CV_I) and interindividual (CV_G) variation.⁷ The

TABLE 129.1 Examples of Analytical Quality Specifications for Imprecision based on Different Models

Analyte	Biological variation observed in dogs $0.5CV_I^{13}$	Derived for veterinary purposes from human regulatory guidelines ²
Erythrocyte count	2.7%	±2%
Hematocrit	3.2%	±2%
Hemoglobin	3.0%	±2%
Leukocyte count	6.1%	±4%
Platelet count	NA	±6%

NA, Not assessed.

derived quality specifications for imprecision and bias are set to keep these at levels negligible compared to the observed biological variation. The general consensus for analytical imprecision (CV_A) is that the desirable level should be below $0.5CV_I$, as this will keep the influence of CV_A on the total variation below 11%.⁵ Criteria for an optimal level ($CV_A < 0.25CV_I$) and a minimum level ($CV_A < 0.75CV_I$) have also been agreed upon.

Quality specifications for analytical bias (B_A) are based on CV_I and CV_G combined, where the criteria are $B_A < 0.125(CV_I^2 + CV_G^2)^{1/2}$, $B_A < 0.25(CV_I^2 + CV_G^2)^{1/2}$ and $B_A < 0.375(CV_I^2 + CV_G^2)^{1/2}$, for optimal, desirable and minimum control, respectively.⁵ This can be combined in a total allowable error (TE_{max}) approach where desirable $TE_{max} < 1.65(0.5CV_I) + 0.25(CV_I^2 + CV_G^2)^{1/2}$.⁶ The total error approach will allow an overall assessment of the quality performance of the test by means of MedX charts plotting observed imprecision and bias in relation to quality specifications (Chapter 130). Table 129.1 includes examples of desirable veterinary quality specifications for imprecision derived from biological variation and human regulatory guidelines, respectively.

Statistical Quality Control (QC) Rules

When analytical quality specifications have been set up for imprecision and bias for a specific analyte, quality control rules must be defined that will detect violations of these specifications with acceptable efficacy.

Control materials, as described above, are used for this purpose. The material is analyzed in 15–20 stable analytical runs to establish a mean and standard deviation of the control material measurements. Control material provided by manufacturers of hematology analyzers or available through other commercial sources often has an assigned interval. If a control measurement falls outside this it is supposed to be rejected. It is highly advisable to establish local values for every control material, as the assigned values and limits may turn out to be sub-optimal for local QC demands, being either too insensitive or resulting in an unacceptable rate of false rejections. As for the use of cut-off values in a diagnostic set-up, there is a close relation between the

sensitivity and specificity of such QC rules, where highly sensitive rules tend to result in increased rate of false-positive rejections of analytical runs and *vice versa* (i.e. less stringent rules being too insensitive to detect critical errors). The task is to identify QC rules that will detect clinically relevant errors (according to the quality specifications) with both an acceptable probability of detecting critical errors and an acceptable risk of false rejection. Furthermore, there are no “golden QC rules” to detect both violation of quality specifications for imprecision and bias with the same efficacy. In our view, unless a method is known to have inherent risk of changing bias (e.g. by poorly defined calibration material), the primary focus should preferably be on implementing QC rules sensitive to changes in imprecision in veterinary hematology, as these will often provide sufficient control of bias also. If globally recognized standards were to be made available for veterinary analyses in the future, assessment of absolute bias would be possible, and the QC focus should be assessed on a test to test basis.

Quality Control rules can be represented by symbols in the format A_L , where A represents statistics or number of control measurements and L identifies the control limit, e.g. 1_{2S} is a control rule with one measurement and a limit of mean $\pm 2SD$ (the standard rule of the Levy-Jennings chart, where an analytical run is rejected if a measurement of the control material deviates from the established mean by more than $2SD$).¹⁹ The optimal rule depends on the quality specifications and whether focus is on detection of unacceptable imprecision or bias. Rules like 2_{2S} and 4_{1S} are sensitive to bias, where 1_{3S} and $1_{2.5S}$ are sensitive to changes in imprecision.¹⁹ Probability of error detection and false positive rates for the different rules can be assessed by power function calculations based on the defined quality specifications and number of control materials used.¹⁹ Based on these assessments the best rule is chosen and applied. The charting of control values over time is not different from the traditional Levy-Jennings chart, it is only the interpretation that is optimized for error detection (Fig. 129.1).

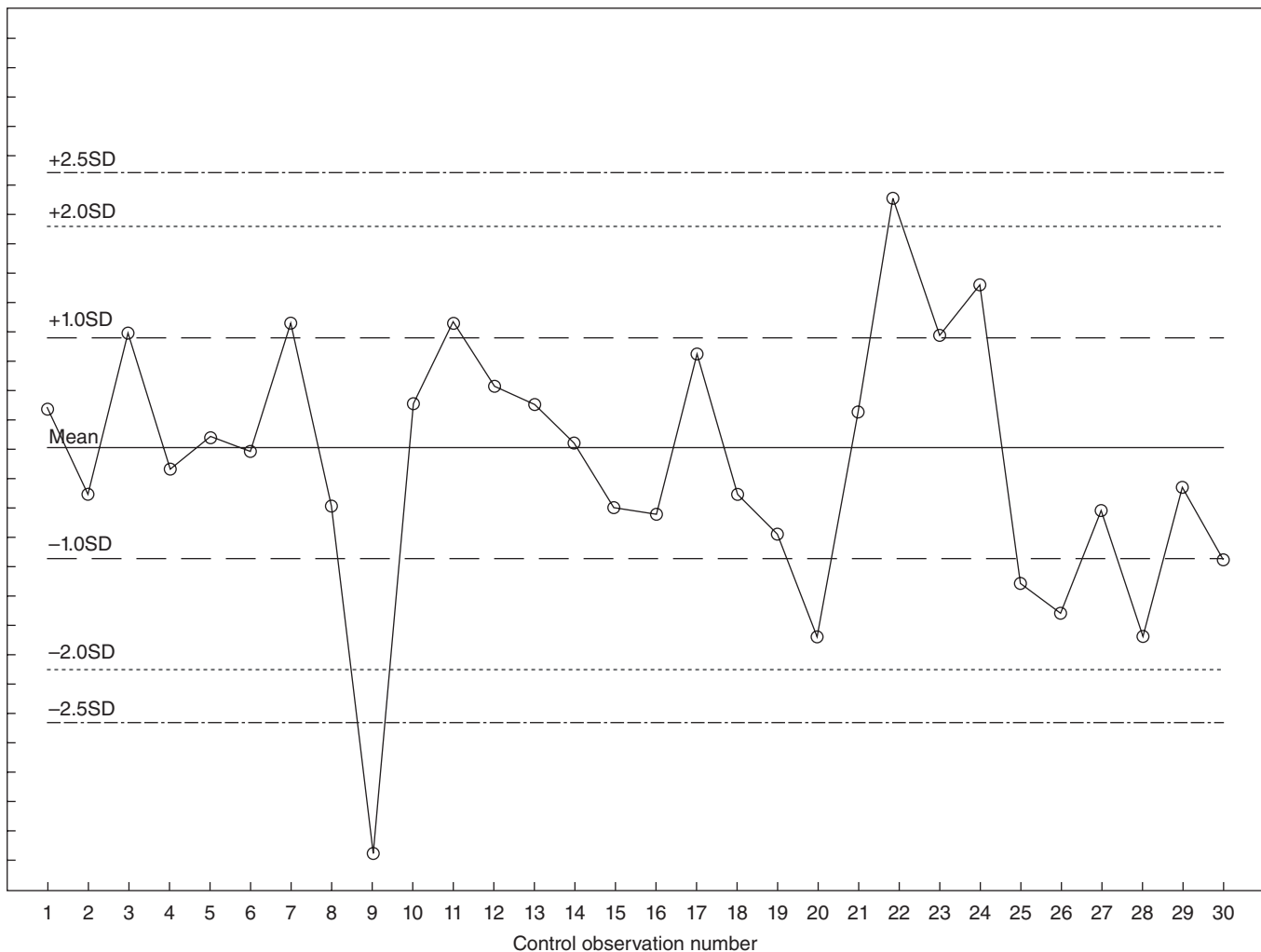


FIGURE 129.1 An example of a chart of 30 consecutive runs of a control material. Mean and standard deviation (SD) have been previously established by 20 measurements during stable performance. Values are interpreted according to chosen Quality Control Rules, e.g. if $1_{2.5S}$ is the rule then a violation is present in run 9; if 1_{2S} is the rule then both runs 9 and 22 are rejected. A rule of 10_x (10 consecutive values on the same side of mean), if applied, is sensitive to a shift in bias. Attention is therefore needed on runs 25–30, which are all below mean.

The standard rule of 1_{2S} usually results in higher, often unacceptably higher, false rejection rates and should only be used when the high sensitivity is needed. This systematic approach for QC optimization has been demonstrated for veterinary purposes also.¹⁵ If both bias and changes in imprecision must be kept under strict control, application of multi-rule QC may be necessary.¹⁹

THE ROLE OF PROFICIENCY TESTING IN QUALITY CONTROL

Proficiency testing programs for veterinary hematology are available and the use thereof is highly advisable and necessary to meet general quality system criteria.⁸ However, as veterinary medicine lacks globally recognized standards for uniform calibration material and as control materials are artificially stabilized for transport in the programs, results are usually only comparable within the same methods. Participation in such programs will facilitate detection of systematic errors not detectable by internal quality control methods, and it may be an indirect way to obtain a crude estimate of bias. Guidelines for assessment of human laboratory tests when proficiency testing is not available exist and some of the recommendations can be relevant for veterinary laboratories also.³ As an example, collaborating with geographically proximate laboratories in a split-sample program may help to identify possible unacceptable differences.

THE ROLE OF MANUAL SLIDE EVALUATION IN QUALITY CONTROL

The manual microscopic evaluation of blood film slides is recognized as a very powerful diagnostic tool in veterinary hematology and its role in routine quality control is no less important.¹ However, the role shifts depending on the analyte in question and the automated methodology used. When the automated methodology is regarded as being more precise and accurate, the main role of blood film evaluation in QC is to confirm or reject results produced by automated analyzers, which can be done objectively by comparison of results (exemplified below under leukocyte differential count). If the automated analyser is regarded as inferior to the manual evaluation or incapable of analyzing the analyte in question, then the manual evaluation is regarded as the main method, and should therefore undergo its own appropriate QC.

White Blood Cell and Red Blood Cell Counts

Manual blood film evaluation will not produce estimates of total red blood cell (RBC) count reliable enough for objective quality control. The same is true for the total white blood cell (WBC) count; however, experienced evaluators will be able to detect marked leukocytosis and leukopenia with the knowledge of the hematocrit

for subjective confirmation of the result from the automated analyzer.

Leukocyte Differential Count

The approved standard for evaluation of instrumental methods for leukocyte differential counts in human medicine is based on manual differentials as a gold standard.⁴ So although a manual 100–200 cell differential count is seemingly mathematically unable to meet analytical quality specifications itself due to sampling variation,¹⁶ it is a convenient qualitative tool to control automated differential counts.

If the automated analyzer used has proven to produce reliable differential counts in the specific species, the quality control will basically consist of detecting gross errors, i.e. systematic misclassification of cell types by the automated differential. The major part of variation in a manual differential count is due to the sampling of low numbers of cells (e.g. 100–200) and a rough estimate of CV_A can thus be derived from the variation of sampling proportions, where $CV_P = [(1 - p)/pn]^{1/2}$.¹⁶

For example, an automated differential reporting 29% lymphocytes is selected for QC (CV_A for the method is 2%). The manual differential ($n = 100$) reports 20% lymphocytes ($CV_A = [(1 - 0.20)/0.20 \times 100]^{1/2} = 0.2 = 20\%$). The combined imprecision is $CV_{\text{combined}} = (0.2^2 + 0.02^2)^{1/2} = 0.201$. The best guess of a true value is the average of the observed values $[(0.29 + 0.2)/2 = 0.245]$ and the maximum difference explainable by the combined imprecision is thus: $\text{Diff}_{\text{max}} = 1.96 \times 0.201 \times 0.245 = 0.10$. With an observed difference of 0.09 = 9%, the run cannot be rejected. Due to the large inherent imprecision of the manual differential count, a difference exceeding what can be explained by the combined imprecision is a rather specific sign of a relevant systematic error.

If an automated differential is rejected, reasons for the misclassification by the automated machine should be identified and corrected, and the sample should be reanalyzed. If reasons are undetectable or uncorrectable, a result based on the manual differential can then be reported if acceptable.

If the automated analyzer has not proven able to produce reliable veterinary differentials, then the manual differential count should be regarded as the method itself, realizing the inherent imprecision of the method. The QC will then shift to consist of analyzing observer agreement.

Leukocyte Morphology

Automated analyzers are in general not yet capable of assessing morphologic characteristics of veterinary leukocytes that exceed leukocyte classification necessary for a differential count (assessment of band neutrophils and toxic changes in neutrophils are good examples). Thus leukocyte morphology evaluation relies on manual evaluation and QC on analyzing observer agreement locally and participation in proficiency testing programs that include leukocyte morphology evaluation.

Reticulocytes

Automated analyzers for veterinary purposes performing reticulocyte counts are available. Quality control of these methods can be performed by comparison of reticulocyte proportions obtained by manual evaluation, as outlined above. If automated methodology is not present and the reticulocyte count is performed manually, the QC will consist of analyzing observer agreement.

Red Blood Cell Morphology

Most analysers based on flow cytometry or impedance-based methodology can produce distributional plots of erythrocyte cell size. Several of the flow cytometry based analyzers also provide the hemoglobin content of individual cells. However, other morphological characteristics may depend on manual recognition and the QC will rely on analyzing observer agreement locally and participation in proficiency testing programs that include erythrocyte morphology evaluation.

Platelets

Platelet clumping is a main pre-analytical reason for falsely low estimates of automated platelet counts, especially in cats where a blood film evaluation is seemingly more reliable in identifying true thrombocytopenia than an automated count.¹⁷ This calls for rigid QC of low platelet counts by manual blood film evaluation, looking for platelet clumps.¹ Some analyzers will flag the presence of platelet clumps based on distribution plots, which is seemingly specific; however, clumps can also be identified in unflagged samples (A.L. Jensen and M. Kjelgaard-Hansen, personal experience).

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Diagnostic Test Validation

ASGER LUNDORFF JENSEN and MADDS KJELGAARD-HANSEN

Test Validation

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Acronyms and Abbreviations

CV, coefficient of variation; DPR, differential positive rate; LOF test, Lack-of-Fit test; LR, likelihood ratio; MEDx chart, method evaluation decision chart; NPV, negative predictive value; P, prevalence; PPV, positive predictive value; r , correlation coefficient; ROC curves, receiver-operating-characteristic curves; SD, standard deviation; \bar{X}_{mean} , mean value; SE, sensitivity; SP, specificity.

A primary function of the laboratory is to minimize the amount of error so that test interpretation, patient care, and/or consumer safety are not compromised. Therefore, a common task in clinical laboratories is to validate a new test or a new analyzer when it is introduced in the laboratory, assuring that test results reflect the status of the animals more than they reflect variation caused by the laboratory test itself.

Validation of diagnostic tests basically concerns reliability (Is the measurement reproducible?) and validity (Does the test measure what it is intended to measure?). This chapter presents practical procedures and statistical tests to investigate common problems in hematology laboratories, such as investigation of imprecision, inaccuracy, method comparison, detection limit and interference. Strategies for investigating how the test works when it is applied to healthy and sick animals, i.e. diagnostic sensitivity and specificity, predictive values and receiver-operating-characteristic curves, will also be outlined.

TEST VALIDATION

The very first step in test validation is to become familiar with the new test or new analyzer, both theoretically and practically. It is often useful to construct a description of the test that includes a description of the analytical procedure, including those materials and equipment necessary for an experienced analyst to obtain a test result (Table 130.1).

The next step is focused on analytical performance. This is followed by observations on how the test behaves when it is actually used on patients (overlap and clinical performance).

Phase 1 – Analytical Performance

Assessment of analytical performance should, as a minimum, include a description of the test and estimates of imprecision, inaccuracy, and detection limits for that test.

TABLE 130.1 Suggested Minimum Information for Diagnostic Tests

- Intended use of the test** – e.g. diagnosing a specific disease among sick animals, monitoring therapy, or screening for a specific disease among healthy animals
- Outline of the analytical principle** – preferably with references to the literature
- Specifications of the analytical instruments and equipment**
- List of reagents** with concentrations in appropriate SI units, source and brand name
- Specimen requirements** – e.g. volume, anticoagulants, and storage
- Full description of all steps in the analytical procedure**
- Calibrating procedure and method for calculating test results**
- Control material** with concentrations in appropriate SI units, source and brand name, control rules applied together with the probability of error detection and probability of false rejection
- Safety precautions** – e.g. when preparing reagents and disposing of waste
- Practicability** – e.g. time needed for analysis of one specimen when the analyst is not prepared for it, costs, and technical skill requirement

Imprecision

Imprecision is a measure of the closeness of a series of measurements of the same material. Imprecision is most often expressed in percent as a coefficient of variation (CV) and is usually calculated as within-run (within-day; intra-assay) and between-run (between-day; inter-assay) imprecision using the standard deviation (SD) and mean value (X_{mean}) in the formula $CV = (SD \times 100\%) / X_{\text{mean}}$. Because imprecision varies with analyte concentration, patient samples with analyte concentrations spanning the analytical range are preferred. Control material can also be used, especially when assessing the between-run imprecision in cases where the analyte is unstable.

Within-Run Imprecision This can be assessed in two ways, either from the differences between duplicate measurements obtained with a number of different samples, or from the results of several replicate measurements made on the same specimen. A practical approach is to select a number of patient specimens (e.g. 30–100) and analyze these in duplicate. The specimens are then divided into three groups (lower, middle and higher end of the analytical range) according to their analyte concentration. The mean value and the number of duplicate pairs in each group are recorded. The differences between the duplicates are used to calculate the standard deviation for each group and eventually, using the mean value of each group, the CV of each group is calculated. A statistical F-test can be used to test whether the SDs differ significantly between the groups. Alternatively, three patient samples with low, middle and high analyte concentrations are measured 10–30 times, in the same analytical run, and the mean value, SD and CV for each patient sample are calcu-

TABLE 130.2 Data on Biological Variation for Some Canine Hematologic Analyses

Analyte	CV _G (%)	CV _I (%)	CV _A (%)	CV _{max} (%)	TE _{max} (%)
Red blood cell count	4.4	5.4	2.8	2.7	6.3
Hematocrit	5.2	6.4	1.1	3.2	7.4
Hemoglobin	4.7	5.9	2.9	3.0	6.9
White blood cell count	12.3	12.1	3.7	6.1	14.4
Albumin	3.0	2.4	1.6	1.2	3.0
Protein	3.1	2.6	1.1	1.3	3.2
Iron	17.2	17.8	0.7	8.9	20.9
Fibrinogen	19.0	17.1	2.8	8.5	20.4

CV_G, Between-dog coefficient of variation; CV_I, within-dog coefficient of variation; CV_A, analytical coefficient of variation; CV_{max}, maximum allowable imprecision; TE_{max}, maximum allowable total error.

lated. A graphical picture of the imprecision (a precision profile) with the CVs on the y-axis plotted against the analyte concentration on the x-axis can be constructed when enough samples have been analyzed.

Between-Run Imprecision Not less than three patient samples with low, intermediate and high analyte concentrations are selected. The samples are analyzed in different analytical runs, e.g. twice daily over 5 days. The number of replicate measurements is recorded and the mean value, SD and CV for each sample are calculated. During the investigation period, the samples should be stored appropriately. If the analyte is unstable, control material can be used instead.

Analytical Goals for Imprecision A common rule of thumb is that imprecision CV should be below 5% for automated assays and below 10% for manual assays. A more objective was formulated by Tonks,¹¹ who suggested that imprecision CV should not exceed one-eighth of the width of the reference interval expressed as a percentage of the mean of the range. More recently, it has been suggested that, based on data on biological variation (i.e. within and between animal variation (CV_I and CV_G; see Table 130.2) the imprecision CV (CV_A) should be less than $0.5 \times CV_I$.³

Inaccuracy

Inaccuracy (also referred to as bias or systematic error) is generally defined as the agreement between the mean value of a series of measurements on the same material and the true value.

Systematic error can be subdivided into constant and proportional systematic error. Constant systematic errors are systematic deviations estimated as the average differences between the two methods. The presence of a constant systematic error indicates that one method measures consistently higher or lower in comparison with the other method. Proportional systematic error means that the differences between the two methods are proportionally related to the level of measurements.

It may be difficult to obtain a species specific specimen with a universally recognized true value and therefore, inaccuracy is often assessed using spiking recovery, linearity checks, control material, and/or comparison of analytical methods.

Spiking Recovery and Linearity Checks

In spiking recovery, the analyte concentration is measured in duplicate in patient samples before and after addition of known amounts of the analyte. Alternatively, patient samples with known analyte concentrations are mixed in various ratios and the analyte concentration in the mixtures is measured in duplicate. The percentages of the measured analyte concentrations to the expected analyte concentrations are then calculated. Ideally, these percentages should be about 100%, usually between 80% and 120%.

In linearity checks, the analyte concentration is measured in duplicate in patient samples before and after dilution with an appropriate diluent (such as physiologic saline or a zero calibrator). The relationship between the measured and the expected analyte concentrations is then investigated by regression analysis of the untransformed and logarithmically transformed data. The confidence interval of the slope of the linear regression equation should include 1, thus indicating that recovery is 100%. When necessary, data are logarithmically transformed to achieve homogeneity of the variances. The confidence interval of the slope of the logarithmic regression equation should include 1, thereby indicating that the recovery is proportional. It also worthwhile to examine whether the data follow a straight line, either by use of a simple Runs-test or by use of the more complex Lack-of-Fit (LOF) test.

Control Material

Inaccuracy can be assessed using control material having a value assigned by the manufacturer. Alternatively, one can use the mean of values assayed in specimens by participants in an external quality assurance scheme. In essence, the control material or the quality assurance specimens are subjected to replicate (e.g. 20–40) measurements. Using a one-sample *t*-test or Wilcoxon Signed Rank test, depending on whether the data are normally distributed, it is tested whether the measured mean (or median) analyte concentration differs from the expected value. The difference between the observed mean (or median) value and the expected mean value is the bias of the analytical method and it can be expressed in percentages of the expected value.

Comparison of Methods

The purpose of a method comparison study is to estimate the type and magnitude of systematic error between two methods and to judge if the two methods are identical within the inherent imprecision of both methods or within preset analytical quality specifica-

tions. A detailed approach to method comparison studies has been described.⁵

Briefly, for quantitative tests the typical protocol includes: (a) obtaining estimates of random error (CV) for both methods, (b) estimating the number of samples to be included in the method comparison experiment, (c) defining acceptable difference between the two methods, (d) analyzing the data, and (e) judging acceptability.

Estimates of random error (CV) are obtained from imprecision studies for both methods. Patient samples (e.g. 30–100) with analyte concentrations spanning the analytical range are analyzed by the two methods. Duplicate measurements on each method are preferred because outlying values are more easily identified and because the data set can be used to calculate imprecision also. The range ratio (i.e. maximum value divided by the minimum value) also indicates the number of samples to be included. If the ratio is low, the number of samples should be high and *vice versa*.

To define the acceptable difference between the two methods, two strategies can be used. The first approach is to calculate the combined inherent imprecision of both methods $(CV_{\text{method1}}^2 + CV_{\text{method2}}^2)^{1/2}$, or in case of duplicate measurements $[(CV_{\text{method1}}^2/2) + (CV_{\text{method2}}^2/2)]^{1/2}$. If the combined inherent imprecision is 10%, and the mean value of the two methods is 200, then the difference between the two methods is expected to be within the interval $0 \pm 1.96 \times CV \times \text{mean}$ in 95% of the measurements, that is $0 \pm 1.96(0.10 \times 200) = 0 \pm 39.2$. The second approach is to specify acceptance limits based on analytical quality specifications usually in the form of either total allowable error (TE_{max}), such as those proposed in Table 130.2, or those reported for human samples by the Clinical Laboratory Improvement Amendments (CLIA) proficiency testing criteria (for more details on CLIA, see <http://www.fda.gov/cdrh/CLIA/index.html>).

For analyzing the data, a plot of the results (or the means of the duplicate measurements) by one method against the other method together with a line of equality is inspected. If the data are linearly distributed and not clumped in one end of the data range, correlation analysis is performed. Then the correlation coefficient (*r*) is calculated. Correlation coefficients (*r*) higher than 0.975 (for data encompassing a small range) or 0.99 (for data encompassing a wide range) indicate that simple linear regression provides useful information about constant error and proportional error via the intercept and slope, respectively. If the intercept differs significantly from 0, then there is a constant systematic error. If the slope differs significantly from 1, then there is proportional error. If *r* is lower than 0.975 (or 0.99), this indicates that more data are typically needed or that other types of regression analysis should be used, e.g. Deming or Passing-Bablok regression techniques.

For judging acceptability, two approaches can be used; acceptability based on inherent combined imprecision or acceptability based on preset analytical quality specification. For the approach based on combined inherent imprecision, a difference plot is constructed by plotting the difference between the methods ($A - B$) on

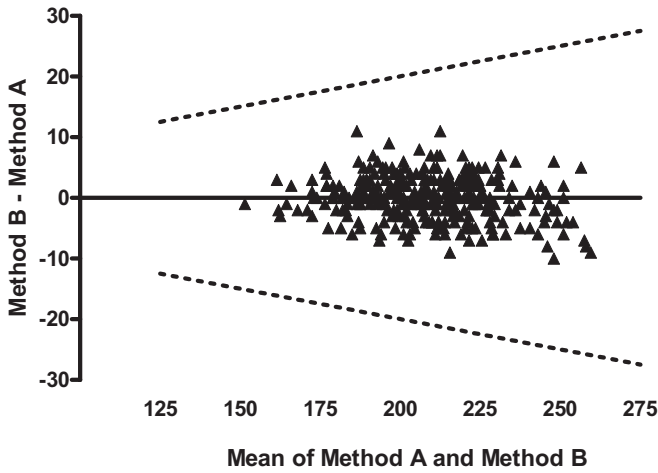
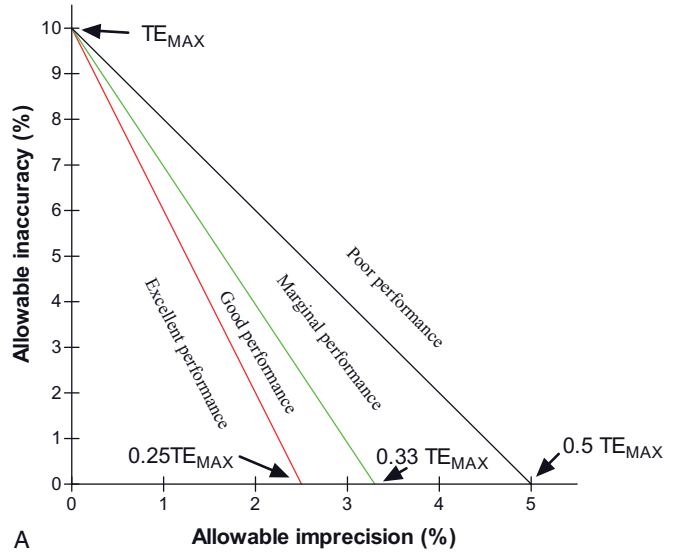


FIGURE 130.1 A difference plot together with lines representing the combined inherent imprecision of two methods. For Method A, CV is 1% and for Method B, CV is 5%. The combined inherent CV of both methods given single measurements is thus 5.1%. At a mean value of 125, the difference between the two methods should be within the interval -12.5 to 12.5 , and at a mean value of 275, the limits are -27.5 to 27.5 . In this case, the two methods are identical within combined inherent imprecision since the differences are symmetrically distributed around zero and all of the differences are inside the dotted lines.

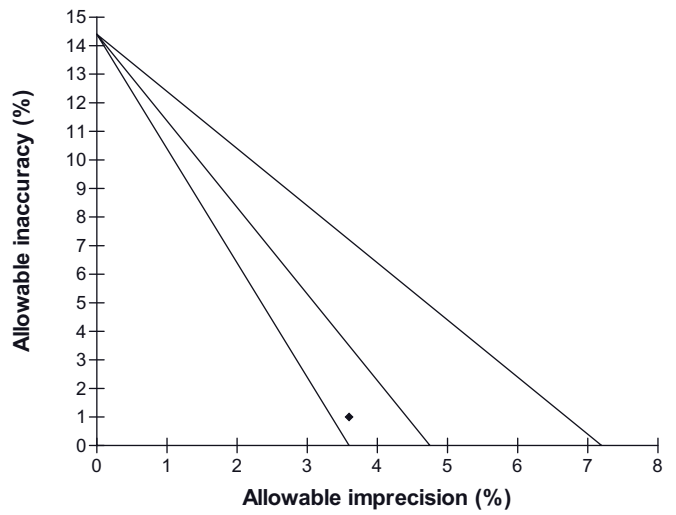
the y -axis against the mean of the methods $[(A + B)/2]$ on the x -axis. Next, the lines representing $0 \pm$ the combined inherent CV at different x -values are inserted in the plot. If the two methods are identical, the differences should be symmetrically distributed around 0, and 95% of the differences should be within the lines (Fig. 130.1). For the approach based on pre-specified criteria, the values for imprecision, inaccuracy and total error are used in a method evaluation decision (MEDx) chart.¹³ This provides a simple graphical tool for comparing imprecision and inaccuracy observed for a test with an analytical quality requirement that is stated in the form of an allowable total error (Figs. 130.2A, B). Assessment can also be based on a six sigma value $\{Six\ sigma = [TE\ (\%) - Inaccuracy\ (\%)]/CV\ (\%)\}$, where values lower than 3 indicate that the methods compare insufficiently.

Detection Limit

The detection limit can be defined as the smallest concentration that the laboratory test, with a stated probability, can distinguish from a suitable blank. A practical approach is to measure a sample with no or a very low content of the analyte (e.g. physiologic saline or a serum sample when investigating an automated blood cell counter) 20–30 times, calculate the mean value and the standard deviation (SD), and estimate the detection limit as mean value (or zero) + 2 (or 3) \times SD. More precise or rigorous calculations are rarely necessary, bearing in mind that the detection limit should serve as a warning limit rather than a justification for continually using the test at its lowest analytical level.



A



B

♦ Observed imprecision and inaccuracy

FIGURE 130.2 MEDx charts for assessing analytical performance. (A) MEDx chart for a test with a maximum allowable total error (TE_{MAX}) of 10%. The lines represent different currently accepted total error criteria: line marked $(0.5 \cdot TE_{MAX})$ represents the total error criterion $TE_{MAX} = Inaccuracy\ (bias) + 2SD$; line marked $(0.33 \cdot TE_{MAX})$ represents the total error criterion $TE_{MAX} = Inaccuracy\ (bias) + 3SD$; line marked $(0.25 \cdot TE_{MAX})$ represents the total error criterion $TE_{MAX} = Inaccuracy\ (bias) + 4SD$. TE_{MAX} can be calculated from data on biological variation or be estimated from CLIA proficiency testing criteria. By plotting the observed inaccuracy and imprecision of a test into its MEDx chart, the analytical performance of the assay can be classified as poor, marginal, good or excellent. (B) MEDx chart for total white blood cell (WBC) count with TE_{MAX} being calculated as 14.4%. Inaccuracy and imprecision were estimated for WBCs on an automated hematology analyzer as 1% and 3.6%, respectively, and using the MEDx chart, it is concluded that the test has good analytical performance.

Additional Analysis of Analytical Performance

High levels of usual blood contents (e.g. lipids, bilirubin, hemoglobin, and glucose) may interfere with the laboratory test⁶ and it may be useful to examine the effects of such substances in greater detail, for instance by comparing the analyte concentration in patient samples before and after addition of the different amounts of the substances. Unconjugated bilirubin and glucose can be purchased as dry matter, hemoglobin can be obtained by freezing of washed erythrocytes, and lipids can be obtained from human parenteral nutrition prescriptions. Different medications and diseases may also interfere in the test, for example, by displacing the analyte from its carrier proteins or by binding important cofactors. In most cases, it is very complicated to examine for potential interference, but significant information can be derived.¹⁵

Although not a topic related to analytical performance *per se*, sources of pre-analytical variation such as storage, type of anticoagulants, medication, venous site of blood sampling, etc., may significantly affect test results, and it is often valuable to investigate these sources.^{7,10}

Phase 2 – Overlap Performance

Initial Application of the Test

The aim here is to compare test results in healthy animals to various groups of diseased animals. Approximately 20–30 animals in each group are tested, and the number of animals in each group, the mean or median values, SD (or 25–75 percentiles), and minimum and maximum values are recorded. A plot of the data, e.g. in a dot diagram, allows for an initial visual assessment of the degree of overlap between the groups. Unpaired *t*-tests or a Mann-Whitney U-test for unpaired observations can be used to test statistically whether test results differ between groups. If a great overlap is detected, the diagnostic value of the assay is usually so low that further evaluation is unnecessary.

It is important to remember that at this initial stage, the prevalence of the disease frequently is unrealistically high, and that animals with the disease in question may be in advanced disease stages that are easier to recognize and thus to include in the study. Furthermore, if the group of animals without the disease in question consists of healthy animals only, then the overlap of test values between animals with and without the disease in question is often further minimized.

Phase 3 – Clinical Performance

In phase 3, the clinical usefulness of the laboratory test is investigated. The outcome of this investigation can be seriously biased by simply incorporating the test under investigation into the evidence used to diagnose the disease in question. Potential sources of bias have been described.⁹ To avoid biasing the outcome of this investigation as much as possible, a blind, prospective and

controlled investigation fulfilling the following criteria should be used.

Selection of Appropriate Animals

The clinical usefulness must be evaluated in the relevant clinical target population, i.e. the population of animals where the disease in question is a possible differential diagnosis and to which the laboratory test is most likely to be applied later. The total number of animals should at least be greater than 40. It is very important to specify the criteria for including animals, especially because animals without the disease in question may have diseases which other users of the test do not consider appropriate differential diagnoses.

Independent Classification of the Selected Animals

The animals in the clinical target population must be classified as being with or without the disease in question independently of the laboratory test being evaluated. Criteria for establishing the diagnosis in question should be specified.

Applying the Test to All Animals in the Study Population

Ideally, but rarely practically, the test should be applied at the same point in the clinical course of the animal's disease. A very thorough description of an appropriate approach for evaluating diagnostic accuracy of tests is given by the *Standards for Reporting of Diagnostic Accuracy (STARD)* steering committee.¹ Other sources of bias possibly introduced by study design and their importance have also been described.²

Sensitivity and Specificity

Test validation is further performed through assessment of the test sensitivity and specificity. Test results are divided into positives and negatives depending on whether or not they exceed a predefined limit, usually the upper or lower limit of the reference interval. The number of positives and negatives for each patient group (with and without the disease) are then arranged in a two-by-two table and sensitivity (SE) (the proportion of patients with the disease that tests positive) and specificity (SP) (the proportion of patients without the disease that tests negative) are calculated as follows: $SE = A/(A + C)$ and $SP = D/(B + D)$.¹⁴

	Disease Present	Disease Absent
Test Positive	A	B
Test Negative	C	D

The reliability of SE and SP depends on the size of the chosen population and it is therefore necessary to provide 95% confidence intervals for SE and SP. These intervals can be calculated using the normal approximation to the binomial distribution, by computer software, or they can be obtained from tables of binomial

distribution. Further, a first line assessment of the test can be performed by calculating Youdens index ($Y = SE + SP - 1$) and in general, the closer this value is to 1, the better the test is.

Predictive Values

For diagnostic purposes, it is of interest to know how likely the disease is when the test is positive and how unlikely the disease is when the test is negative. This information is provided by the positive predictive value (PPV) (or predictive value of a positive test or post-test probability of disease following a positive test result) and the negative predictive value (NPV) (or predictive value of a negative test or post-test probability of no disease following a negative test result).¹² Positive predictive value and NPV can be calculated using the same two-by-two table used for calculation of SE and SP if the proportion of diseased animals reflects the disease prevalence in the group of animals to which the test is to be applied. Positive predictive value is then calculated as $[A/(A + B)]$ and NPV is calculated as $[D/(D + C)]$, and 95 % confidence limits can be calculated as for SE and SP. In many cases, the proportion of diseased animals in the two-by-two table does not reflect the prevalence of the disease in the target animal population and therefore, PPV and NPV are calculated for disease prevalences (P) varying from 0 to 1 using the following formulas:

$$PPV = (SE \times P) / \{(SE \times P) + [(1 - P)(1 - SP)]\}$$

and

$$NPV = [SP(1 - P)] / \{[SP(1 - P)] + [P(1 - SE)]\}.$$

Positive predictive value and NPV can then be plotted in a graph with PPV and NPV on the y -axis and P and the x -axis. At low disease prevalences, PPV is low, and at high disease prevalences, PPV is high. The clinical approach to increased disease prevalence of the suspected disease is to obtain a history and perform a clinical examination before applying the test. In contrast, NPV is maximal at low disease prevalences and a negative test result therefore appears to be most useful for ruling out the disease at low disease prevalences.

Receiver-Operating-Characteristic (ROC) Curves

By changing the cut-off value by which test results are divided into negatives and positives, SE and SP change so that when SE increases, SP decreases and *vice versa*. The cut-off value is varied over the spectrum of test results and the resulting pairs of SE and SP are plotted in a graph (a ROC curve) with SE on the y -axis and $(1 - SP)$ on the x -axis (Fig. 130.3).¹⁶

The ROC curve is used to assess the overall diagnostic accuracy of the test. This can be done visually because the better a test discriminates between diseased and non-diseased groups, the closer to the upper left-hand corner of the graph is its ROC curve situated. More formally, the area under the ROC curve (W) and the standard error of the area (SE_W) can be calculated using

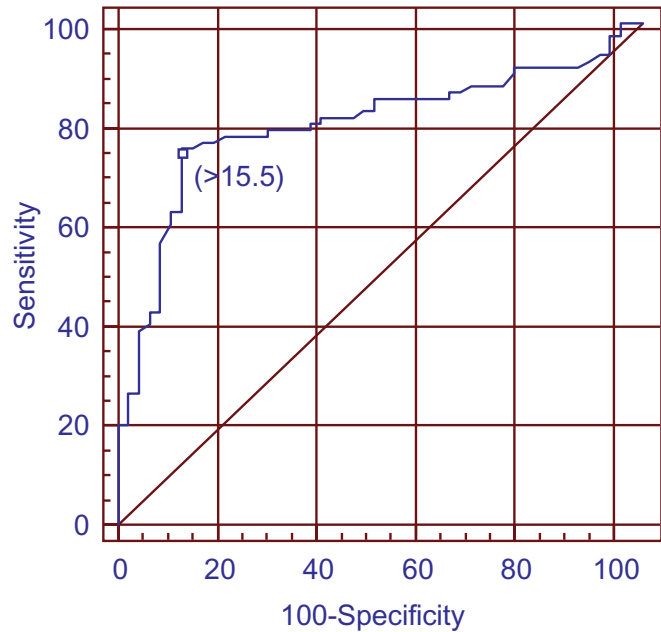


FIGURE 130.3 Receiver Operating Characteristic (ROC) curve. A ROC curve is constructed by plotting sensitivity as a function of $(1 - \text{specificity})$ over the range of selected cut-off values. Tests with poor performance tend towards the diagonal line. Tests with good performance tend upward and leftward. The highest sensitivity and specificity is the top-leftmost point and the cut-off value that gives this point in this plot is 15.5.

the trapezoidal rule or a computer program. The area is then compared to 0.5, which is the area under the ROC curve of a worthless test that does not discriminate between the two patient groups, by calculating a Z value [$Z = (W - 0.5) / SE_W$]. Z values above 1.96 indicate that the area under the ROC curve is significantly different from 0.5 and that the test is different from a worthless test. Two or more tests used to discriminate between two patient groups can also be compared using their ROC curves. Again, a visual assessment can be used or the areas under the ROC curves can be compared statistically. The latter is most often done by using a computer program.⁴

The ROC curve is a plot of SE and SP pairs for different cut-off values. From the list of SE and SP corresponding to each cut-off value, it is possible to identify the cut-off value associated with the highest SE and SP simply by calculating the differential positive rate (DPR) for each cut-off value from the formula $DPR = [SE - (1 - SP)]$. The cut-off value with the highest DPR is also the cut-off value associated with the highest SE and SP.

Likelihood Ratios

The ROC curve is a plot of the effect of changing the cut-off value on SE and SP. This dynamism can be described by the slope (or tangent) of the ROC curve at any single, defined, point corresponding to the cut-off value of interest. Two slopes have been defined: $[SE /$

$(1 - SP)]$ and $[(1 - SE)/SP]$. The former is called the likelihood ratio of a positive test result (LR+), and the latter is called the likelihood ratio of a negative test result (LR-). Another definition for LR+ is the probability of obtaining a positive test result in an animal with the disease divided by the probability of obtaining a positive test result in an animal without the disease. In other words, a LR+ of 4 means that a positive test result is four times as likely to occur in a diseased animal as in a non-diseased animal. Likewise LR- is defined as the probability of obtaining a negative test result in an animal with the disease divided by the probability of obtaining a negative test result in an animal without the disease. As a general rule, a good LR+ exceeds 2 whereas a good LR- is near 0. Because likelihood ratios are derived from SE and SP, it is necessary to determine the confidence intervals of the ratio, which is a somewhat complicated procedure involving χ^2 statistics.⁸

Diagnostic test validation can be taken to yet another and more complicated phase where outcome and utility assessment are addressed. In this phase, it is assessed if a laboratory test is clinically useful by whether it successfully answers a question of consequence to patient management. To assess if measurements have a positive consequence on the case management of individuals or groups of individuals, it can be assessed if the test improves the outcome of a clinical problem, if it provides equivalent clinically useful information faster than established parameters, if it provides equivalent clinically useful information more cost-effectively than established parameters, or if measurements are done on material more easily obtained or with better stability than established methods. The difficult part here is to set criteria for outcome and quality of clinically useful

information, and this is perhaps the reason why outcome and utility assessment is rarely done.

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Reference Intervals

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Relevance of Reference Intervals
Establishment of Reference Intervals (*de novo*)

Transference of Reference Intervals
Partitioning of Reference Intervals

Acronyms and Abbreviations

CLSI, Clinical and Laboratory Standards Institute; CV_i , intra-individual variation; CV_g , inter-individual variation; IFCC, International Federation for Clinical Chemistry and Laboratory Medicine; ROC, receiver operating characteristics.

The diagnostic work-up of veterinary patients is a decision-making process. Decisions of “ruling out” or “ruling in” differential diagnoses are based on the achieved level of clinical knowledge, and the use of laboratory tests (including hematology) is an integral part of this process. Relevant tests are performed to change pre-test probability of a diagnosis to a post-test probability level, facilitating “ruling in” (increased post-test probability of a diagnosis) or “ruling out” (negligible post-test probability of a diagnosis) of one or more of the differential diagnoses. To make this decision-making process consistent, established decision criteria are essential. Optimally, these are objective and optimized to the specific clinical situation to limit false decisions with the risk of false diagnoses. Examples of specific “decision limits” can be found in human medicine, such as those defined by consensus agreement for cholesterol. A major challenge for such decision limits to be applicable across laboratories and methodologies is to ensure precision across both. One of the major prerequisites for this is traceability of calibration material to generally recognized standards, such as the CRM470 standard for human serum proteins established by the International Federation for Clinical Chemistry and Laboratory Medicine (IFCC).¹ However, such programs are rare, if not absent, in veterinary clinical laboratory medicine, making establishment of “decision limits” primarily a local task, comparable only across laboratories using the exact same methodology, at best.

Clinical decision limits are in nature dependent on the clinical objective and thus applicable for specific clinical situations only. Hence, the end result will be a

demand for a vast number of decision limits in veterinary laboratory medicine. This task has not yet been encountered and decisions are therefore most often made by means of the limits of population-based reference intervals based on a distribution of reference values obtained from a reference sample group of healthy individuals. That is, the population-based reference interval describes the variation of values that can be expected in healthy animals of the population from which samples are submitted to the laboratory. The logical reasoning behind using such intervals is that a measured value outside the reference interval is unlikely to originate from a healthy individual, and will thus represent a diseased individual. This reasoning makes the use of population-based reference intervals independent of the clinical objective and more globally applicable. However, many pitfalls in this reasoning exist and population-based reference intervals should only be seen as guidelines when no better means are available⁶ and not assimilated to a “clinical decision limit” optimized for a specific clinical situation.¹⁶ The widespread lack of proper clinical decision limits in veterinary laboratory medicine should therefore be seen as an opportunity for future studies, such as studies involving Receiver Operating Characteristics (ROC) curve analysis.^{8,12} Meanwhile, it should be ensured that the population-based reference intervals are applied appropriately, and the remainder of this chapter will describe different aspects of this topic. A joint guideline from the Clinical and Laboratory Standards Institute (CLSI) and IFCC on “Defining, establishing, and verifying reference intervals in the clinical laboratory” has recently been released in a new

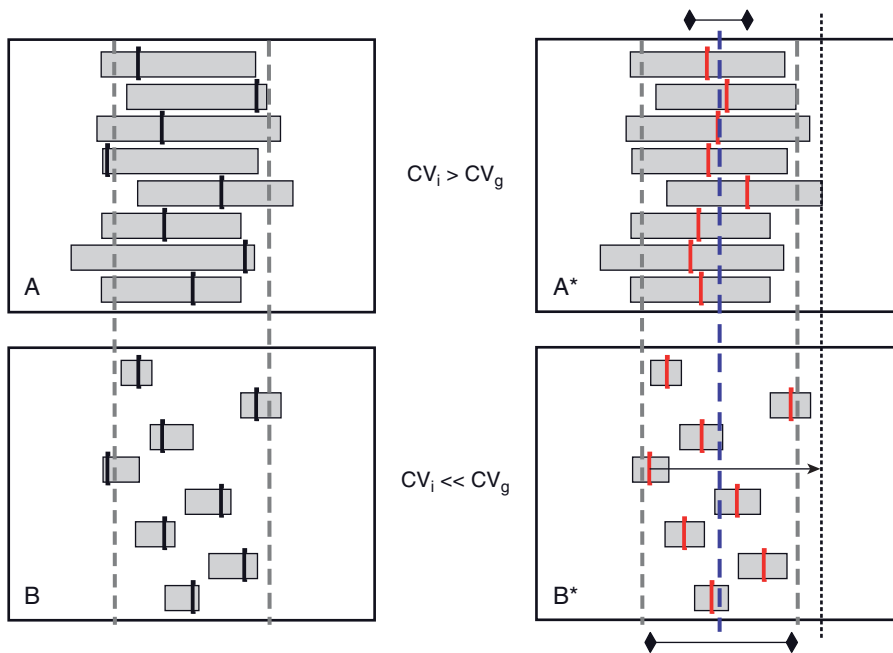


FIGURE 131.1 Single sampling of individuals in a reference sample group will not reveal the nature of biological variation, including intra- (CV_i) and inter-individual (CV_g) variation, respectively. Very different CV_i (shaded bars in A [large CV_i] and B [small CV_i]) can result in identical reference distributions of reference values (black lines) and thus in identical reference limits (broken shaded lines). It can be appreciated that the distribution of homeostatic set-points (red lines, A* and B*) are much further from the population mean (blue broken line) and with a wider range (diamond-ended lines, A* and B*) when $CV_i << CV_g$ (B*). As a result, the upper reference limit becomes an insensitive mean to detect changes, as a merely abnormal analytical result (dotted line, B*) is highly abnormal for several individuals (arrow, B*). In this case other means than population-based reference intervals should be considered for detection of changes.

edition.³ Working groups from the American Society of Veterinary Clinical Pathology (ASVCP) and the European Society of Veterinary Clinical Pathology (ESVCP) are currently preparing a similar joint guideline for veterinary purposes (based on the CLSI C28-A3) expected to be released in the near future⁷ (M. Kjelgaard-Hansen, personal communication). The guideline is a welcome opportunity to standardize how reference intervals are applied in veterinary medicine and should be applied as widely as possible.

RELEVANCE OF REFERENCE INTERVALS

The *de novo* establishment of a reference interval of high quality is very demanding, e.g. in terms of resources and manpower, and it is therefore appropriate to assess the mere relevance of a reference interval before taking on the task. Obviously, if clinical decision limits exist, as described above, these should be used and a reference interval is unnecessary. Information on biological variation of the analyte should also be considered before reference values are determined.

The width of a reference interval is mainly determined by the combined analytical, intra-individual (CV_i) and inter-individual (CV_g) variation. Analytical variation is routinely assessed and controlled by method validation and quality control. On the other hand, CV_i and CV_g remain unrevealed unless specifically designed studies are applied to the population.⁵ The ratio (CV_i/CV_g), also referred to as the index of individuality, is highly relevant for assessing the relevance of a reference interval. The ratio between CV_i and CV_g cannot be detected by traditional single sampling of healthy individuals, as identical distributions can arise from very different ratios of these (Fig. 131.1). If CV_i is much smaller than CV_g , there is a marked individuality, and

a population-based reference interval becomes an insensitive means of detecting pathological changes in the individual (Fig. 131.1). A ratio below 0.6 (which is not uncommon for human analytes) is generally accepted as a criterion defining population-based reference intervals as generally insensitive, and other means for detecting changes should be considered.⁵ Data on biological variation in veterinary species are still limited, but such data are useful for setting standards in method validation (Chapter 130), quality control (Chapter 129) and for the assessment of applicability of reference intervals. Further studies in this area are highly warranted, and detailed descriptions on study design are available.⁵

If it is decided that a reference interval is needed and relevant for a specific analyte, then different options are available, including (1) *de novo* establishment of a reference interval, and (2) transference of a reference interval from another source.

Each option will be described below, with emphasis on the reference interval transference as this solution will be the most relevant to many veterinary laboratories, due to the cost and effort needed to accomplish *de novo* reference intervals.

ESTABLISHMENT OF REFERENCE INTERVALS (DE NOVO)

One of the key issues of the recent CLSI guideline (C28-A3) is how to establish a reference interval, and this guideline together with the upcoming ASVCP/ESVCP guidelines on the same topic should be consulted before starting up the process.³ A 13-point protocol outlines the task (Table 131.1), which was recently assessed to be directly transferable for veterinary purposes.² It is emphasized that the primary focus should be on

TABLE 131.1 Protocol Outline for Obtaining Reference Values and Establishing Reference Intervals³

1. Establish a list of analytical interferences and sources of biological variability from medical and scientific literature
2. Establish selection (or exclusion) and partition criteria and an appropriate questionnaire designed to reveal these criteria in the potential reference individuals
3. Execute an appropriate written consent form for participation in the reference interval study and have the reference individual complete the questionnaire (veterinary modification: Owner's consent and completion of questionnaire)
4. Categorize the potential reference individuals based on the questionnaire findings and results of other appropriate health assessments
5. Exclude individuals from the reference sample group based on the exclusion criteria or assessments indicating a lack of good health
6. Decide on the appropriate number of reference individuals in consideration of desired confidence limits
7. Prepare, properly and consistently, the selected individuals for specimen collection for the measurement of a given analyte consistent with the routine practice for patients
8. Collect and handle the biological specimen properly and in a manner consistent with the routine practice for patient specimen
9. Collect the reference values by analyzing the specimens according to the respective analytical methodology under well-defined conditions and consistent with the routine practice for patients
10. Inspect the reference value data and prepare a histogram to evaluate the distribution of data
11. Identify possible data error and/or outliers
12. Analyze the reference values, i.e., select a method of estimation and estimate reference limits and the reference interval
13. Document all of the previously mentioned steps and procedures

sampling an appropriate reference sample group (Fig. 131.2), preferably by a direct *a priori* method, where criteria for selection, exclusion and partitioning are set before blood sampling is performed. Second, a direct *a posteriori* sampling method can be used with the only difference being that selection, exclusion and partitioning criteria are applied after actual sampling. However, the criteria have been criticized to be too theoretical and demanding,¹⁰ which is probably the reason why they have been largely ignored in both human and veterinary clinical pathology.

A more tempting method for veterinary purposes is the indirect *a posteriori* sampling method, making use of databases and samples collected for other purposes, e.g. blood donors, health screening and preoperative samples for elective surgery. Although special care should be taken not to include large numbers of unhealthy individuals, the method has been used successfully for both veterinary and human purposes.^{4,17} Collection, handling and analysis of the samples to obtain reference values should be done under condi-

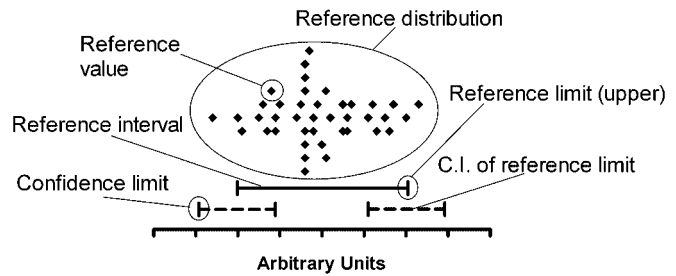


FIGURE 131.2 Nomenclature used in the establishment of reference intervals. Reference individuals (e.g. healthy dogs) are defined within the general population to comprise a reference population from which is selected a reference sample group on which are determined reference values (diamonds). These values characterize the reference distribution from which are calculated reference limits that may define the reference interval. Reference limits are determined with a certain degree of variability that can be expressed by a confidence interval (C.I.) of the reference limit, delineated by confidence limits.

tions according to a well-defined, but routine practice under which the reference interval will later be used.

Traditionally, much focus is put on selection of statistical methods to analyze reference value data, but the number one priority in *de novo* establishment of reference intervals is to fulfill and document the sampling of the reference sample group and use of analytical methodology to obtain values. Only on a distant third priority is the choice of appropriate statistical methods to analyze the obtained data. The reason for this is that no post-sampling statistical procedures can correct for poor sampling of reference individuals, and that different choices of statistical procedures will make little difference if the reference values are of a high quality.

Statistics include outlier detection, where visual examination of a frequency distribution is a very useful tool that can be supported by other objective techniques. However, unless outliers are known to be aberrant observations, the emphasis should be on retaining rather than deleting them.³ If the distribution of reference values is Gaussian or can be transformed to fit a Gaussian distribution, the reference interval can be assessed by parametric methods.^{3,5} As the assumptions made to perform the parametric statistics are easily violated, the IFCC recommends making use of non-parametric statistics if sampling of at least 120 reference individuals is managed. A reference interval corresponding to the 95% confidence interval is easily obtained by the non-parametric approach; the n reference values are ranked and the interval is then delineated by ranks r_1 and r_2 , being $r_1 = 0.025(n + 1)$ and $r_2 = 0.975(n + 1)$. Thus, in theory, a reference interval can be assessed by non-parametric methods with as few as 39 samples (as this is the lowest value of n where r_1 and r_2 will reach integer values, namely $r_1 = 1$ and $r_2 = 39$). However, it is still recommended to include at least 120 data points to assess the precision of the established reference limits.³ Finally, an iterative method ("the robust method") has been described offering a relevant alternative to the non-parametric approach

when $n < 120$. In short, it works by initial location of median and spread of observations, and through an iterative process actual observations are down-weighted according to their distance to the central tendency. For detailed instructions on computation, which can be performed in common spreadsheets, original literature should be consulted.¹¹ This method can be used for skewed distributions also.

TRANSFERENCE OF REFERENCE INTERVALS

As mentioned above, at the local laboratory level, *de novo* establishment of reference intervals is largely ignored by both human and veterinary laboratories due to the associated necessary cost and efforts to do so. Here transference offers a valid alternative, which can be done appropriately by sampling only 20 reference individuals.

Transference can be done at different levels, i.e. within-laboratory (when substituting old methodologies with new ones) or with other laboratories or manufacturers as donors; however, the procedures and tools are basically the same:

1. Choose reference interval to transfer
2. Validate reference interval
3. If unsuccessful: transform reference interval by means of method comparison, if appropriate
4. Validate transformed reference interval
5. If unsuccessful: then only *de novo* establishment is left.

When choosing an external reference interval donor, the main focus should be on methodology and population used to establish the donor reference interval. The relevance of the donor reference interval increases the more these factors match, and so does the chance of a successful validation.

The validation step consists of a sequential comparison of 20 reference values with the donor reference

interval, where the donor reference interval is accepted if two or fewer reference values fall outside the donor reference interval. If three or four fall outside, the procedure must be repeated with an extra 20 reference values, with the same criteria for acceptance. If unsuccessful again, then the donor reference interval is rejected (Fig. 131.3A), which is also the case if five or more values fall outside in the primary comparison.

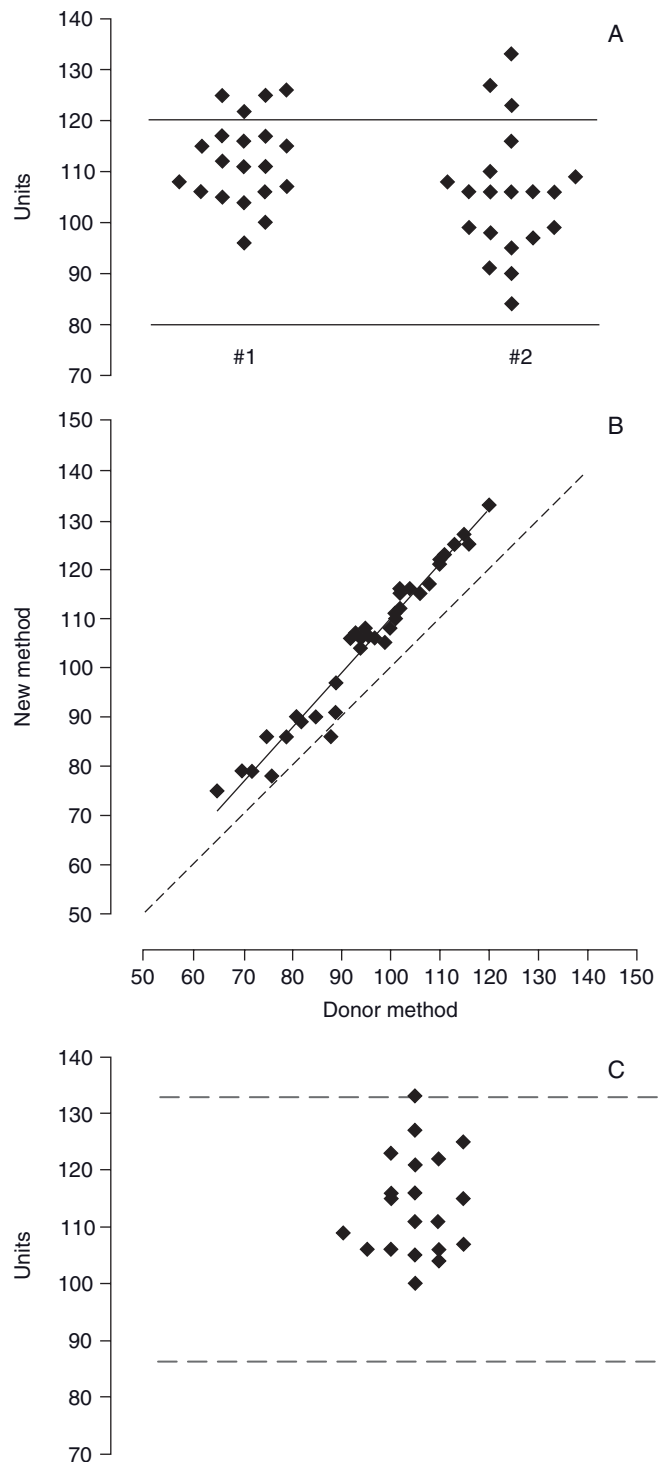


FIGURE 131.3 Validation of transferred reference intervals can be performed by sequential comparison of reference values (diamonds, $n = 20$) to the donor reference interval (black lines). If less than two values fall outside the reference interval, the donor reference interval is accepted. However, if three or four values fall outside (A, #1) the process must be repeated with the same criteria (A, #2) with a final rejection if two or more fall outside (which is the case here). If five or more fall outside in the primary comparison it will result in a final rejection of the donor reference interval. A method comparison can reveal and assess systematic bias to transform the donor reference interval to fit local methodology (B). In this case, the linear regression analysis result was $Y = 1.11X - 1.3$, $r^2 = 0.96$, and with 1.3 (Y -intercept) being numerically low compared to the donor reference interval [80–120] the reference limits were transformed by the regression equation [$1.11 \times 80 - 1.3$; $1.11 \times 120 - 1.3$] to the new interval [87.5–131.9]. Subsequently, the transformed reference interval must be validated as described, in this case with success as less than two reference values fall outside (C). The dotted line in (B) represents the line of agreement between methods ($Y = X$).

If the donor reference interval is from another laboratory, or the transference is between methodologies within the same laboratory, then the discrepancy may be due to a systematic bias that can be corrected by transformation. This is obviously not feasible if the donor reference interval originates from the manufacturer of the method used. Such bias can be revealed and assessed by thorough method comparison (Fig. 131.3B).¹³ Care should be taken to ensure that a distribution of samples spanning the entire reference interval is used for the method comparison (these do not have to be reference samples). If a systematic bias is identified and the Y-intercept of the regression line is small compared to the reference interval, both the upper and the lower reference limit should be transformed by the regression equation (Fig. 131.3B). The transformed reference interval must subsequently be validated as described above (Fig. 131.3C). If validation is unsuccessful, further reference values must be obtained to establish the reference interval *de novo*.

PARTITIONING OF REFERENCE INTERVALS

The global applicability of a reference interval in a population of individuals is based on the assumption of homogeneity in the population, which is an ongoing challenge to control. If homogeneity is violated in a clinically important manner, it is necessary to identify the subgroup causing the violation and create separate reference intervals for the subgroup. Several examples of such veterinary subgrouping already exist; e.g. polycythemia in Greyhounds¹⁵ and inherent idiopathic asymptomatic thrombocytopenia in Cavalier King Charles Spaniels.¹⁴ Clearly, more unidentified subgroups exist in the veterinary species than documented at the present time, and here the task is to be vigilant and take action when signs of subgroups appear, such as frequent outlying observations from a certain breed. Most often, the discrepancy is unidirectional (outlying observations are either systematically higher or lower than established limits) which is a sign of different means of the subgroup and general population (difference in variation would cause bidirectional discrepancy, but are infrequent and will not be described here). Several guidelines exist to determine whether the discrepancy is of clinical significance; for example, (1) if the difference between subclass means exceeds 25% of the width of the combined reference interval¹⁸ or (2) if

more than 4% of the individuals in the subgroup falls outside the established reference interval.⁹ If a clinically important discrepancy is identified, a separate reference interval should be established *de novo* for the subgroup, as described above.

Current examples of existing partitioning of reference intervals are mainly based on phenotypically recognizable groupings, e.g. sex, breed, etc.; however, future partitioning may also include genotypical grouping, if these are to be characterized routinely on an individual level.

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Evaluation of Bone Marrow

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Bone Marrow Collection in Dogs and Cats

- Indications and Contraindications
- Collection of a Bone Marrow Aspirate versus Biopsy
- Techniques and Locations of Bone Marrow Collection
 - Bone marrow collection in dogs
 - Bone marrow collection in cats

Bone Marrow Collection in Horses

Bone Marrow Collection in Ruminants

- Bone Marrow Collection in Cattle
- Bone Marrow Collection in Sheep
- Bone Marrow Collection in Llamas
- Bone Marrow Collection in Camels

Bone Marrow Collection and Processing in Laboratory

- Animals
- Sampling Procedures

Flow Cytometry Staining Techniques

- Cytology Preparations
 - Materials for push smear bone marrow cytological preparation
 - Cytologic cytopsin slides from flow cytometric samples
- Staining Procedure for Cytologic Preparations
- Slide Preparation and Staining of Clinical Bone Marrow Samples
 - Slide Preparation
 - Staining
- Evaluation of Bone Marrow Smears

Acronyms and Abbreviations

BM, bone marrow; BSA, bovine serum albumin; CD, cluster of differentiation; CD71, transferrin receptor; CD45, leukocyte common antigen; DioC6, dihexyloxacarbocyanine; EDTA, ethylenediaminetetraacetic acid; FACS, fluorescence activated cell sorting; FITC, fluorescein isothiocyanate; FL, fluorescence; FSC, forward scatter; EDTA, ethylenediaminetetraacetic acid; MDS, myelodysplastic syndrome; MGG, May-Gruenwald-Giemsa stain; M:E ratio, myeloid erythroid ratio; Nm, nanometer; LDS, laser dye styryl; PBS, phosphate buffered saline; PE, phycoerythrin; RBC, red blood cell; SSC, side scatter; WBC, white blood cell.

BONE MARROW COLLECTION IN DOGS AND CATS

Indications and Contraindications

Bone marrow (BM) examination is indicated for detection of abnormalities that are not recognized during the routine evaluation of a peripheral blood smear. Moreover, bone marrow collection should be performed in animals with suspected lymphoproliferative or myeloproliferative disease, myelodysplastic syndrome (MDS), or diagnosis and staging of hematopoietic tumors such as lymphoma, histiocytic neoplasia, multiple myeloma, or metastatic carcinoma (especially mammary carcinoma), and sarcoma. Unexplained hypercalcemia and fever of unknown origin also may

raise the suspicion of paraneoplastic syndrome due to a tumor affecting the BM. Bone marrow collection is indicated in the diagnostic workup of unexplained, persistent cytopenias including nonregenerative anemias, neutropenia especially in the absence of a left shift, assessment of regeneration in thrombocytopenic animals, as well as bicytopenias or pancytopenias. The presence of abnormal cell populations in the blood, such as blasts, rubricytes in the absence of regeneration, atypical morphology of leukocytes, red blood cells (RBCs), or platelets, is also an indication for evaluation of bone marrow.

Contraindications for bone marrow aspiration include severe coagulopathies. Thrombocytopenia, however, is not a contraindication for bone marrow collection because significant hemorrhage is rarely a

problem. The risk of bone marrow infection is negligible.¹⁵ The most important contraindication, however, is performing a bone marrow aspirate that is unnecessary.⁵

Collection of a Bone Marrow Aspirate versus Biopsy

During bone marrow aspiration for preparation of a smear, architecture of bone marrow tissue is destroyed. The advantage of this technique is that single cells are evenly spread on a slide which allows excellent evaluation of cellular morphology. Thus, bone marrow aspiration is indicated in all diseases which affect the whole bone marrow so that the aspiration is representative for the whole marrow. These include hyperplasia or hypoplasia of cell lineages, leukemia, and myelodysplastic syndromes.¹⁵ The advantage of a biopsy is that the architecture of the bone marrow remains intact so that the relation of cellular populations to each other and to stromal cells can be evaluated.

Indications for bone marrow biopsy include:¹⁵ (1) a bone marrow aspiration which is repeatedly not successful (*punctio sicca*); (2) non-diagnostic bone marrow cytology (e.g. bone marrow aplasia or hypoplasia); and (3) focal abnormalities (e.g. myelofibrosis, granulomatous inflammation, multiple myeloma, bone marrow necrosis, metastatic carcinoma). Because the need for bone marrow biopsy frequently cannot be predicted at the time of bone marrow aspiration, some clinicians routinely collect both samples.

Techniques and Locations of Bone Marrow Collection

Bone Marrow Collection in Dogs

Locations for bone marrow collection in dogs include the iliac crest (Fig. 132.1) and the anterior site of proximal humerus. In large dogs, the third, fourth, or fifth sternebra may be aspirated. In toy breeds with a small



FIGURE 132.1 Demonstration of iliac crest site for bone marrow collection in a dog.

iliac crest, the trochanteric fossa of the femur can be used. Use of the sternum is associated with risk of penetrating the thoracic cavity and injuring intrathoracic organs. The iliac crest and proximal humerus are the most frequently used sites in dogs. An advantage of bone marrow aspiration at the proximal humerus is that the bone is not covered by fatty tissue, which makes marrow aspiration easier at this site in obese dogs. Moreover, there is a lower risk of hemorrhage at this localization.

Generally, BM aspiration from the iliac crest can be performed in standing, awake dogs. For BM aspiration from the proximal humerus, the dog is positioned in lateral recumbency. In anxious or aggressive dogs, sedation or general anesthesia is required. The skin over the site of collection is shaved and aseptically prepared. Approximately 1–2 mL of a 2% lidocaine solution is used for local anesthesia of all tissue layers, especially the periosteum. A 16–18 gauge \times 2.5 cm Klima Rosegger, Rosenthal or Illinois sternal needle with an interlocking stylet is used for bone marrow aspiration.

For aspiration at the iliac crest, the greatest prominence of the wing of the ileum is palpated. The needle is inserted with slow rotating movements in an angle 45° parallel to the long axis of the ileum. Reaching the bone marrow may cause a brief pain reaction that confirms the location of the needle. After removal of the stylet, a 10 mL syringe is attached and approximately 0.5 mL of bone marrow is aspirated. Aspiration of a larger amount should be avoided because it results in blood dilution of the marrow specimen. Avoiding use of an anticoagulant generally yields the best results of staining; however, smears have to be prepared immediately after sampling as bone marrow coagulates rapidly. For some indications such as analysis with a hematology analyzer² or fluorescence activated cell sorting (FACS), the collection syringe can be rinsed with 2–3% ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. EDTA solution can be prepared by injecting 0.35 mL of sterile saline in a commercially available evacuated EDTA blood collection tube that contains approximately 1.5 mg EDTA.⁵ For several flow cytometric protocols, heparin is the anticoagulant of choice. However, heparin anticoagulated marrow should not be used for preparation of smears for cytologic examination as it causes significant staining artifacts. After bone marrow collection, hemorrhage is stopped by applying several layers of gauze with firm pressure for a few minutes to the aspiration site.

With exception of the sternum, core biopsy specimens are taken at the same sites as aspiration samples. The core biopsy is performed with an 11–13 gauge Jamshidi needle, a cylindrical needle with a tapered distal end and beveled cutting tip. The needle is inserted into the bone as described for the bone marrow aspiration. However, the stylet is removed after touching the cortex and the needle is advanced approximately 0.5–1 cm into the marrow cavity with an alternating clockwise-counterclockwise motion to obtain an adequate specimen. Then, the needle is removed a few millime-

ters and advanced again in a slightly different direction to secure the specimen in the needle. Finally, it is pulled out with a rotating movement. The core biopsy specimen is removed by introducing a probe through the distal cutting end. The sample is placed in neutral, buffered 10% formalin solution for histologic processing.

Bone Marrow Collection in Cats

Bone marrow collection in cats is from the iliac crest, the proximal humerus, and, occasionally from the trochanteric fossa of the femur. In cats, general anesthesia is frequently required. To obtain bone marrow aspirates from the iliac crest, the cat is positioned in sternal recumbency with hind limbs tucked alongside the body. Straight positioning is important to get bone marrow from the iliac crest. The overall collection technique is comparable to the procedures described in dogs.

BONE MARROW COLLECTION IN HORSES

The proximal rib, tuber coxae, and sternum are sites for bone marrow collection in horses.^{19,22} The proximal rib contains active marrow throughout life. The rib is an easy site to reach but has several disadvantages. One disadvantage is that the bone is relatively thick and difficult to penetrate in aged horses. Another disadvantage is that the rounded surface of the rib makes it difficult to seat the needle into the bone and increases the risk of slipping off the rib and puncturing the pleural cavity. This can result in pneumothorax. The sternum is a reliable and frequently used site for obtaining bone marrow. The sternum is not covered by a large muscle mass and the bone is relatively thin at this site. Disadvantages of this site are that the sternum is awkward to reach and that the site is near the heart. The tuber coxae are only used for obtaining marrow in foals and horses less than 4 years old.¹⁹ When the tuber coxae are used, the needle is inserted into the midportion of the tuber coxa and directed toward the opposite coxofemoral joint.

Bone marrow aspiration and core biopsy are performed in standing patients. The amount of sedation and restraint varies depending on the temperament of the patient. The skin over the site should be clipped and aseptically prepared. All tissue layers, including the periosteum, are anesthetized with a 2% lidocaine solution. A 15-gauge \times 5 cm styletted Jamshidi bone marrow aspiration/biopsy needle (also called a Janus® needle) is preferred for marrow aspiration. A stab incision is made in the skin and the needle is inserted until it reaches the bone. The bone is penetrated using a slow rotational movement until the needle is firmly seated in the bone. For sternabrae, the needle is then advanced 1–2 cm further into the marrow cavity. For bone marrow aspiration, the stylet is removed and a 6 mL syringe (that can be rinsed with 2% EDTA solution) is attached. One to several quick, forceful, aspirations physically

detach marrow particles and minimize hemodilution. However, forceful aspiration can result in pain with resultant jumping or kicking. An 11 gauge \times 10 cm Jamshidi biopsy needle is used to obtain core biopsy specimens. The biopsy needle is advanced into the bone until it reaches the cortex. The stylet is removed and the needle is advanced 1–2 cm further into the marrow cavity. The needle is then rotated forth and back several times to loosen attachments and then is withdrawn with a rotating motion.

BONE MARROW COLLECTION IN RUMINANTS

Bone Marrow Collection in Cattle

Bone marrow aspiration smears and core biopsy specimens from adult cattle have been obtained from the sternum and dorsal end of the ribs.^{11,23} The iliac crest is an unsatisfactory site because of the anatomy of the bovine pelvis. A Turkel® trephine needle (Trephine Instruments Inc., USA) has been used for bone marrow aspiration from the rib.¹¹ Major disadvantages of this technique include the effort needed to penetrate the bone cortex in aged animals and the risk of puncturing the pleural cavity resulting in pneumothorax. The adjustable depth control on the Turkel trephine needle helps to prevent penetration of the pleural cavity but does not eliminate the risk.

The preferred site for bone marrow collection in adult cattle is the sternum.^{23,25} A 15 gauge \times 5 cm styletted Jamshidi bone marrow aspiration/biopsy needle is preferred for marrow aspiration. An 11 gauge \times 10 cm Jamshidi biopsy needle is used to obtain core biopsy specimens. Cows should be sedated before bone marrow aspiration. Intravenous xylazine provides adequate sedation. The aspiration site is the 3rd or 4th sternebra. This site can be located by counting the ribs starting with the last one; however, the location on the sternum coincides with an imaginary line joining the olecranon. With the cow in a standing position, a small area over the sternum is shaved and aseptically prepared and all layers of tissue, including the periosteum, are anesthetized with a 2% lidocaine solution. A stab incision is made in the skin and the Jamshidi needle is inserted (with stylet in place) until it reaches the bone. The bone is penetrated using a slow rotational movement until the needle is firmly seated in the bone. The needle should be advanced approximately 1–1.5 cm. One can frequently sense when the needle enters the marrow cavity because of a sudden reduction in resistance.

In calves, the proximal humerus is an excellent site for marrow collection. Calves can be restrained in lateral recumbency and the method described for dogs and cats can be followed. A 15 gauge \times 5 cm styletted Jamshidi or Illinois sternal biopsy needle with the needle guard removed can be used for aspiration and an 11 gauge \times 10 cm Jamshidi needle can be used for core biopsies.

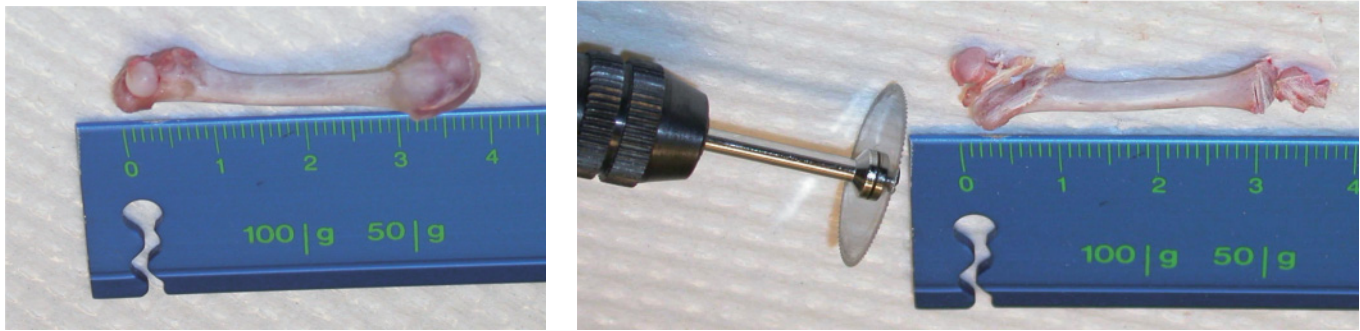


FIGURE 132.2 Bone marrow sampling procedure from the femur in rodents.

Bone Marrow Collection in Sheep

The sternum is the preferred site for bone marrow collection in sheep.⁶ Sheep are restrained in a sitting position by an assistant who holds a foreleg in each hand. The 3rd or 4th sternebra is located by following the rib to its articulation. The site is aseptically prepared and anesthetized as described for cattle. A 15 gauge \times 5 cm styletted Jamshidi or Illinois sternal biopsy needle with the needle guard removed can be used for aspiration. For bone marrow aspiration, the depth of penetration from the skin surface varies between 2.5 and 3.8 cm and bone penetration is approximately 0.5 cm. An 11 gauge \times 10 cm Jamshidi needle can be used for core biopsies.

Bone Marrow Collection in Llamas

The sternum is the preferred site for bone marrow collection in llamas.¹ Adult llamas are sedated with xylazine and can be placed in a chute or in left lateral recumbency. The 3rd or 4th sternebra is located by following the rib to its articulation. The site is aseptically prepared and anesthetized as described for cattle. A 15 gauge \times 5 cm styletted Jamshidi or Illinois sternal biopsy needle with the needle guard removed can be used for aspiration. An 11 gauge \times 10 cm Jamshidi needle can be used for core biopsies.

Bone Marrow Collection in Camels

Collection of bone marrow has been described from the dorsal rib of dromedary camels.¹⁷ Camels were sedated by intravenous administration of xylazine. A site over the 5th through 8th dorsal rib was aseptically prepared and anesthetized as described for cattle. The bone marrow needle and details of the technique were not described.

BONE MARROW COLLECTION AND PROCESSING IN LABORATORY ANIMALS

In recent years, bone marrow analysis in toxicological clinical pathology generally involves flow cytometric analysis as the primary means of bone marrow evalua-

tion, and cytologic preparations to assess morphologic changes and abnormalities in maturation or proliferation. This approach permits quantitative analysis of bone marrow, and includes total nucleated cell counts, differential cell counts, and a myeloid:erythroid (M:E) ratio. Flow cytometry counts 10,000 cells or more, thus providing a much faster and more accurate estimate of cellular populations than microscopic assessment.

Sampling Procedures

In rodents (rat, mouse and hamster), one of the femurs is removed at necropsy and trimmed of muscular tissue. The femoral head and the distal epiphysis are cut off using a small electrical saw (Fig 132.2). In the dog and cynomolgus monkey, the fifth rib is removed at necropsy and trimmed of muscular tissue. A piece of the rib (1 cm) is cut from the articulating end with the costal cartilage (Fig. 132.3). The bone marrow tissue is flushed, approximately five times, with 2 mL PBS containing 50% fetal bovine serum using a needle and syringe (Fig. 132.4). The bone marrow cell suspension is washed and resuspended in ice-cold physiologic buffered saline (PBS) containing 0.5% bovine serum albumin (BSA). The nucleated cell count is determined using a hematology analyser and the cell concentration is adjusted to 10^7 cells/mL.

Flow Cytometry Staining Techniques

Saad et al. described a method for rat bone marrow differential cell counts using a combination of two monoclonal antibodies for the differential expression of leukocyte common antigen (CD45) and transferrin receptor (CD71).²⁰ This was coupled with the side scatter for cellular complexity and nucleic acid staining with LDS-751 for separation of nucleated cells from mature red blood cells (Fig. 132.5). A similar approach can also be used for monkey and mouse bone marrow analysis. Briefly, bone marrow cells are labeled with fluorescein isothiocyanate (FITC)-conjugated anti-CD45 and phycoerythrin (PE)-conjugated anti-CD71 monoclonal antibodies. The tube is incubated in an ice bath in the dark for 20 minutes followed by a washing step with PBS containing 0.5% BSA. The cell pellet is resuspended in

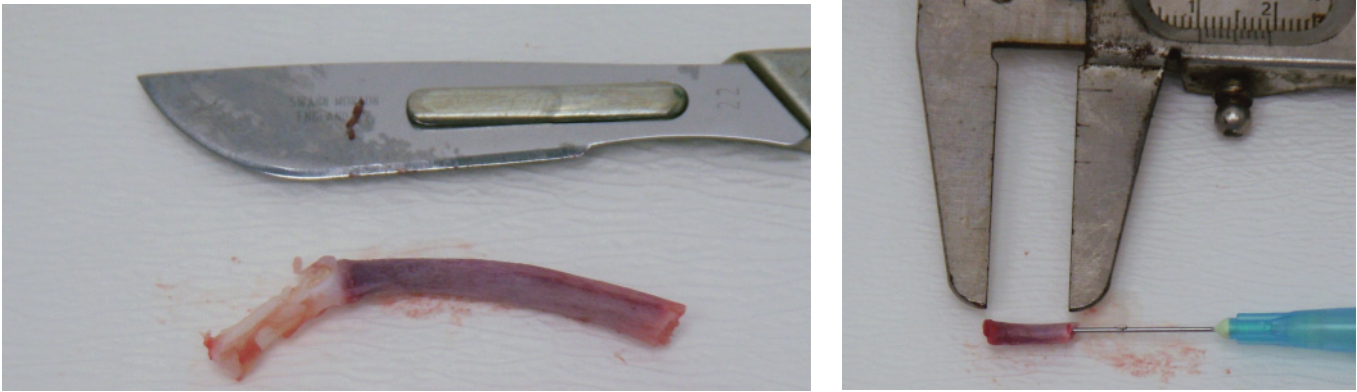


FIGURE 132.3 Bone marrow sampling procedure from the rib in dog or monkey.



FIGURE 132.4 The flushing of bone marrow from the piece of rib (dog and monkey) or from the femur of rodents.

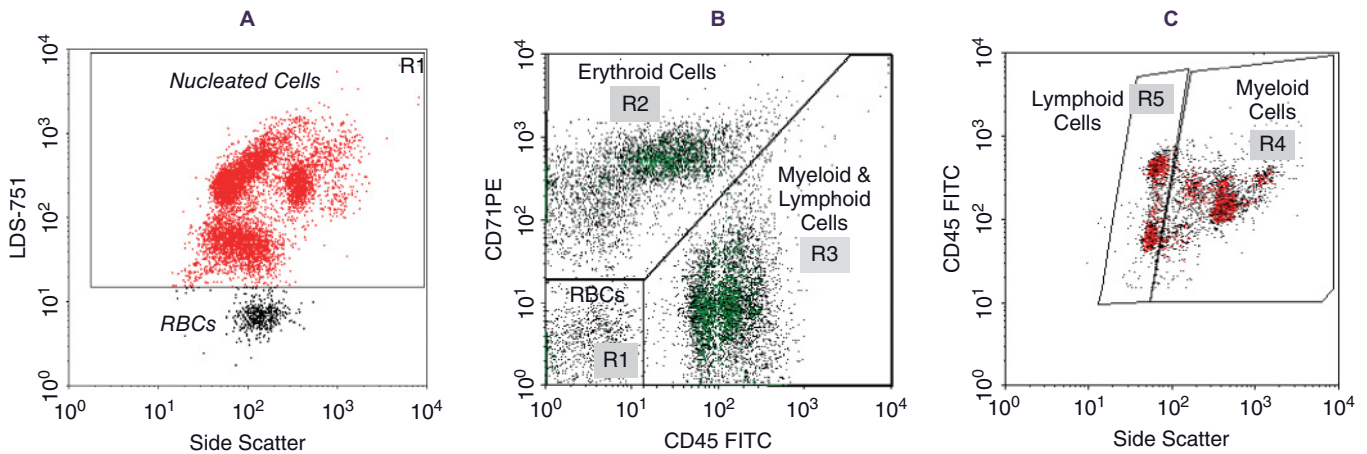
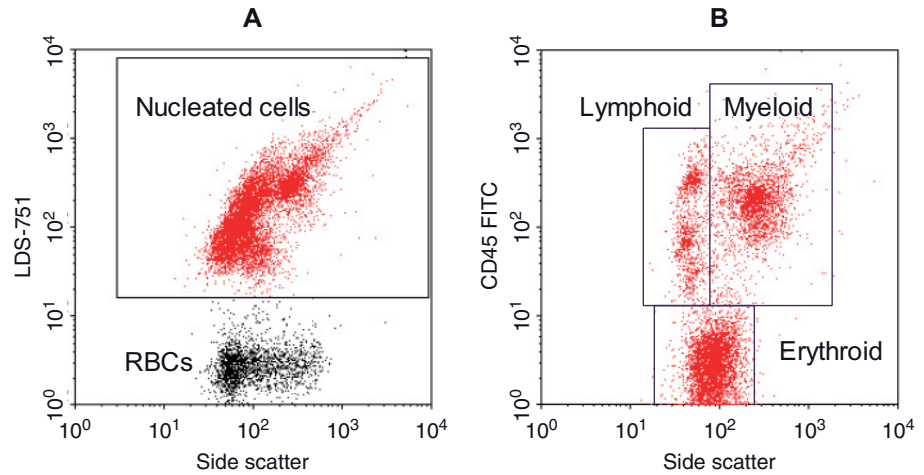


FIGURE 132.5 Rat bone marrow differential analysis using combination of CD45:FITC, CD71:PE and LDS-751 staining. Cytoqram C represents events in gate R3 of cytoqram B.

0.4mL of cell fixative (1% formaldehyde). For at least 20 minutes before analysis of samples, 20µL of LDS-751 staining solution is added. The LDS-751 staining solution is prepared by diluting 20µL of stock solution (1mg/mL methanol) with 3mL PBS. The analysis is performed in a flow cytometer using an excitation

wavelength of 488nm from an argon ion laser. Data can be collected from 10,000–20,000 cells from each sample in list mode to save forward scattered (FSC) values and side scatter (SSC) in a linear scale, and FL1 (green), FL2 (orange) and FL3 (red) in a logarithmic scale. A cytoqram of CD45 versus SSC can be plotted on all LDS

FIGURE 132.6 Dog bone marrow differential analysis using combination of CD45:FITC, cellular complexity (SSC) and LDS-751 staining. Cytogram B represents events in nucleated cells gate (cytogram A).



751-positive cells (nucleated cells) on a cytogram of LDS 751 versus SSC. Standardized gates can be used for all files and the percentages of erythroid, lymphoid and myeloid cells determined. The dog bone marrow analysis is based on cellular complexity and the expression of CD45 in combination with LDS-751 staining (Fig. 132.6).

A few other approaches on the use of flow cytometry for bone marrow analysis of laboratory animals are also described in the literature. For rodents, Martin et al.¹² described a flow cytometric method based on membrane potential using 3,3'-dihexyloxycarbocyanine iodide (DioC6) staining (Fig. 132.7). A combination of 2',7'-dichlorofluorescein-diacetate staining (indirect myeloperoxidase staining) and monoclonal antibodies for lymphocyte subpopulations (OX52 for T-lymphocyte and OX4 for B-lymphocyte) was used by Criswell et al.³ for the differential analysis of rat bone marrow (Fig. 132.8). Flow cytometric methods using cellular complexity and light scatter properties in combination with the expression of CD71 or CD45 have been described for rat²¹ and dog²⁴ bone marrow evaluation. More recent work by Zhao et al.²⁶ described a method for rat bone marrow analysis using the combination of CD3, CD45R, CD71, and CD11b/c in combination with the DNA dye DRAQ5.

Cytology Preparations

The femur is the preferred site for bone marrow sampling of rats and mice. Usually the femur is removed promptly at necropsy and longitudinally split using a bone cutter. The following procedure allows preparation of push smear preps which allow optimal preservation and concentration of cells.¹⁸ Alternatively, cytospin preparations can be prepared from flushed femur samples prepared for flow cytometric analysis.

Materials for Push Smear Bone Marrow Cytological Preparation

Reagents for push smear cytological preparation include: immunological grade bovine albumin, 22%,

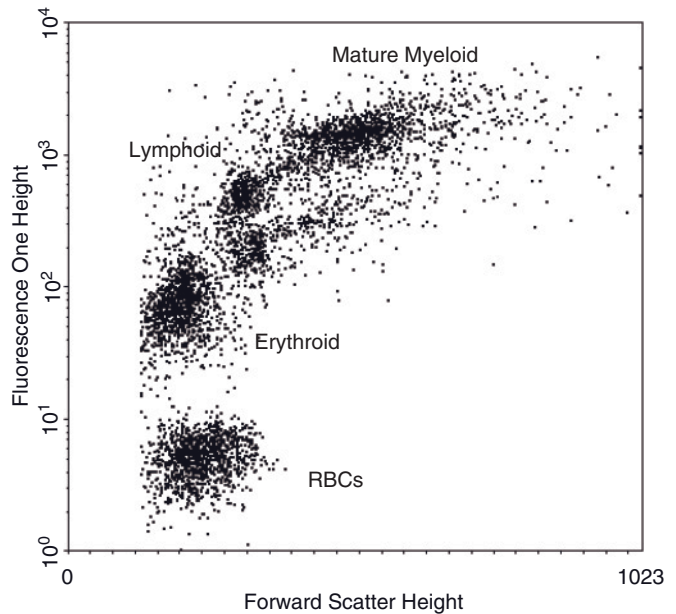


FIGURE 132.7 Rat bone marrow differential analysis using DioC6 staining.

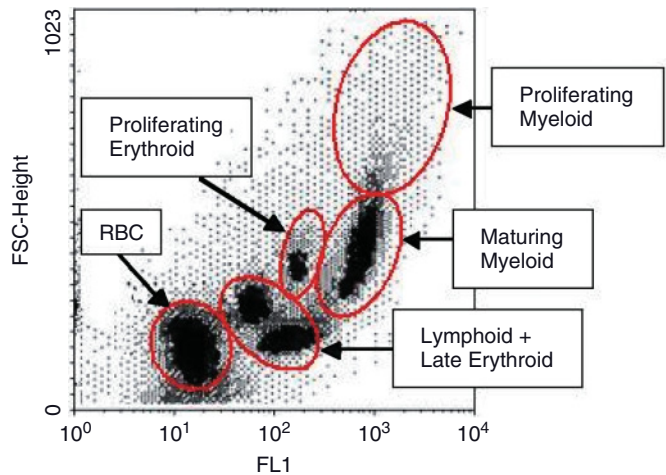


FIGURE 132.8 Rat bone marrow differential analysis using 2',7'-dichlorofluorescein diacetate staining (indirect myeloperoxidase staining).

polymerized (Median Diagnostics), several pieces of 4 cm × 4 cm square parafilm, bone cutter for sampling of longitudinally sliced femur, scissors, paintbrush no. 0 or 1 regardless of species, and glass slides. The following procedure is used:

1. Place a drop of bovine albumin fluid on several pieces of parafilm square
2. For rodent femur preparations, cut the femur lengthwise, using sharp tools
3. Pick out as much bone marrow as possible with the brush; place on parafilm next to the drop of bovine albumin fluid
4. Dip brush in albumin fluid and mix into the bone marrow to make a uniform solution
5. Place a drop of this bone marrow on 4–5 slides
6. Place another slide gently on top of the slide containing the sample and spread slides apart
7. Air dry.

Cytologic Cytospin Slides from Flow Cytometric Samples

For cytospin slides, take 100 µL of the bone marrow suspension prepared for flow cytometry and add 400 µL PBS containing 0.5% BSA. From this diluted cell suspension, 100 µL is placed in the funnel of a cytocentrifuge. The specimen is centrifuged at 50g for 3 minutes and the slide is removed and air dried.

Staining Procedure for Cytologic Preparations

Wright-Giemsa or May-Gruenwald-Giemsa (MGG) staining provides optimal contrast for bone marrow cytology preparations. Rodent bone marrow requires slightly longer staining time with Giemsa staining compared with MGG stain for the best differentiation between nucleated RBCs and lymphocytes. The Giemsa stain needs to be freshly prepared just before use and care should be taken to maintain a pH of 6.8 for the phosphate buffer.

SLIDE PREPARATION AND STAINING OF CLINICAL BONE MARROW SAMPLES

Slide Preparation

Several techniques for preparing bone marrow smears in a clinical veterinary setting have been described. The most commonly used techniques are:

1. Preparation of a wedge-type smear (no anticoagulant needed)
2. Drop-squash or particle-squash preparations (require EDTA as anticoagulant).

For preparation of a wedge-type smear, several drops of the aspirated bone marrow are ejected onto a microscope slide and a spreader slide is backed into the area containing concentrated bone marrow particles. Then it is lifted off and placed on a clean slide for prepara-

tion of a wedge-type smear. Generally, up to 10–12 slides should be made.¹⁵ For the drop-squash preparation, a drop of bone marrow is placed on a slide and gently squashed by a second longitudinally-placed slide or coverslip.¹⁶

Particle-squash smears are prepared from 2 drops of bone marrow that are placed on a glass slide. The slide is subsequently tilted so excess blood is drained away. Marrow particles, seen as white specks in the red marrow, can be picked up with a microhematocrit capillary tube using capillary action and are carefully transferred onto a second slide so that squash preparations can be made.¹⁶ EDTA-anticoagulated bone marrow can also be placed in a Petri dish for collection of bone marrow particles.

Staining

After preparation, bone marrow smears are quickly air dried. Routine staining is performed with Romanowsky-type stains (May-Gruenwald-Giemsa, Wright stain). It is important to remember that bone marrow smears require a longer staining time because they are thicker and the material is often unevenly distributed. Fast stains like Diff Quik®, however, are less reliable because they do not stain chromatin structure as clearly as May-Gruenwald-Giemsa or Wright stains. Additional stains may be needed so that unstained slides should be saved. Cytochemical staining methods are discussed in Chapter 144.

EVALUATION OF BONE MARROW SMEARS

Unlike evaluation of bone marrow in laboratory animals, clinical evaluation of bone marrow is qualitative. Thus, only the proportions of cellular populations in relation to each other, their maturation, and cellular morphology are reported. For interpretation of these cytological findings, clinical signs and hematological results of the patient must be considered as they indicate the actual demand for RBCs, white blood cells (WBCs), and platelets in the blood. For instance, bone marrow with normal cellularity and a M:E ratio within the reference interval would be considered normal for a nonanemic animal but hyporegenerative for an animal with a severe anemia and no reticulocytosis.

Criteria to be evaluated in bone marrow smears include: (1) estimation of cellularity; (2) assessment of megakaryopoiesis; (3) evaluation of bone marrow iron (not in cats); (4) M:E ratio; (5) maturation cellular lineages (megakaryopoiesis, erythropoiesis, granulopoiesis, lymphopoiesis); (6) presence of dysplastic changes; (7) evaluation of other cell types (plasma cells, mast cells, macrophages, osteoclasts, osteoblasts; and (8) evidence of neoplastic cells or parasites.

While cellularity, megakaryopoiesis, stainable bone marrow iron, and the presence of atypical cell clusters are evaluated at 100× magnification, M:E ratio, maturation of cells and cellular morphology are assessed at 500× to 1000× magnification. Cellularity is estimated in

bone marrow spicules by comparing the percentage of cells to the proportion of fat tissue (cell-to-fat ratio). Normal cellularity ranges between 25% and 75% cells.⁷ Cellularity is age-dependent: bone marrow spicules of growing animals contain approximately 75% cells, those of young adults 50%, and marrow spicules of older animals 25% cells. The number of hemosiderophages in the bone marrow spicules is indicative of the amount of stored bone marrow iron. Stainable bone marrow iron is increased in chronic inflammation and immune-mediated anemia; however, it is decreased in iron deficiency and in newborn or young animals.⁴ In cats, however, bone marrow iron may not be readily apparent upon staining.

Quantification of megakaryopoiesis is considered to be difficult due to the irregular distribution of megakaryocytes.¹⁰ Thus, the megakaryopoiesis should be evaluated on low power fields where the platelet precursors can be easily identified. The quantification of megakaryopoiesis can be performed by counting the number of megakaryocytes in 70–100 low power fields (100× magnification) and three slides.^{15,16} Mean counts of 2.76 megakaryocytes/low power field and 1.55 megakaryocytes/low power field were obtained in one study, using drop squash and particle squash preparations, respectively.¹⁶ Counting megakaryocytes in 20–25 spicule-containing fields achieves an acceptable variance of 20%.¹⁴ However, the percentage of megakaryocytes is highly dependent on the technique of smear preparation and quality of the bone marrow films.¹⁶ Therefore, megakaryopoiesis is routinely only semi-quantitatively assessed. Generally, bone marrow spicules contain several megakaryocytes.⁵ Normally, >70% of megakaryocytes are mature.⁷ A percentage of promegakaryocytes of more than 50% suggests regeneration.⁵ A predominance of promegakaryocytes is consistent with an early stage of regeneration or a maturation defect.

Proportions and maturation of erythroid, myeloid, and lymphoid cellular lineages are examined at 500× to 1000× magnification. For determination of the M:E ratio, a 500–1,000 cell differential count is recommended. Because particles have a greater percentage of granulocytes, 50% of cells from bone marrow particles and 50% from the inter-particle regions of the smear should be classified.⁵ A differential cell count is indicated to document slight to moderate increases in blast cell counts that may differentiate myelodysplastic or low grade leukemic conditions from benign conditions. Alternatively, a subjective M:E ratio can be determined from the cells seen during cytological evaluation of the bone marrow smear.⁵ The normal M:E ranges from approximately 0.5:1 to 2–3:1 in healthy dogs,¹³ horse, cow, sheep and pigs,⁹ however, broader ranges of up to 4–5:1 have been described.^{8,10} A M:E of 1:2 has been reported in healthy cats.⁹ Interpretation of the M:E requires consideration of bone marrow cellularity: a normal M:E in face of hypercellular marrow indicates an equal increase in both myelopoiesis and erythropoiesis. An increased M:E may be due to either a decrease

in erythropoiesis or an increase in granulopoiesis, whereas a decreased M:E suggests either a granulopoietic hypoplasia or an erythropoietic hyperplasia.

The maturity of cells in the erythroid and myeloid series should also be evaluated. Normally more than 85% of cells in the granulocytic (i.e. metamyelocyte, band, and segmented cells) and erythroid (i.e. rubricyte and metarubricyte) lineages should be in the mature non-proliferative pool. Causes of immaturity in these lineages include leukemias, myelodysplastic syndromes, and immune-mediated hemolytic anemia in which erythroid precursor cells are destroyed at a particular stage of maturation.

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Hematopoietic Cell Culture

CHRISTINE S. OLVER

History of Hematopoietic Culture
Types of Hematopoietic Culture
Progenitor Assays

Long Term Bone Marrow Culture
Long Term Culture Initiating Cell Assay
Growth Factors Used in Hematopoietic Culture

Acronyms and Abbreviations

BFU-E, burst-forming unit-erythroid; BMMC, bone marrow mononuclear cells; CFU, colony-forming unit; CFU-E, colony-forming unit-erythroid; CFU-GEMM, colony-forming unit-granulocyte, erythroid, macrophage, megakaryocytic; CFU-GM, colony-forming unit-granulocyte/macrophage; EPO, erythropoietin; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte/macrophage-colony stimulating factor; HC, hematopoietic cell; LTBM, long term bone marrow culture; LTC-IC, long term culture initiating cell.

Hematopoietic cell (HC) culture involves the cultivation of primary cells; those that have been derived directly from the host. Sources of HC include bone marrow, peripheral blood mononuclear cells, spleen (rodents) and fetal liver (rodents). Generally, HC culture is used to study progenitor cells, which are lineage committed but morphologically indistinguishable cells. Because progenitors cannot be identified microscopically, their descendants need to be studied and identified in order to retroactively identify the progenitor. This is done by culturing in a semi-solid medium under conditions that allow a single progenitor to form a clone of differentiated cells. Thus, progenitor cells are also called colony-forming units (CFU). Hematopoietic cell culture can enumerate these progenitor cells in a crude suspension of hematopoietic tissue cells. Besides enumeration, HC culture is used to study the physiology, differentiation mechanisms and growth factor control of hematopoiesis. Additionally, liquid based HC culture is employed to establish long-term cultures that recapitulate the microenvironment of *in vivo* hematopoiesis. It is also used to expand either lineage-committed cells or undifferentiated stem cells for transplantation. This chapter describes the history of HC culture, a description of its methods and uses, the types of resulting cells, and the growth factors required.

HISTORY OF HEMATOPOIETIC CULTURE

The discovery of progenitor cells in hematopoietic tissue occurred almost 50 years ago⁸ using both *in vitro* and *in vivo* assays. The colony-forming unit-spleen (CFU-S) assay was originally developed in the early 1960s^{7,9} as a way to identify and enumerate bone marrow progenitor cells. The CFU-S assay provided important information on the nature of both hematopoietic progenitor cells and less differentiated (and more pluripotent) hematopoietic stem cells. Progenitor/stem cells from bone marrow mononuclear cells (BMMC) injected into lethally irradiated mice migrate to the spleen and form macroscopic colonies of variably differentiated hematopoietic cells. One cell is capable of forming a colony of 10^6 differentiated cells, indicating that the original cell is capable of massive division as well as differentiation. These assays provided evidence that these cells are of low frequency in bone marrow suspensions (1 per 10^4 bone marrow cells), have no distinctive morphologic features, have extensive proliferative and differentiation capacity, and are capable of limited self-renewal.

In vitro clonal progenitor assays were developed shortly after CFU-S assays.^{1,5} Initially only granulocytic progenitors were cultured, but then conditions for erythroid colony formation were developed. A liquid

culture system permitting long term culture of bone marrow progenitors with an adherent stromal feeder layer, termed long term bone marrow culture was first developed by Dexter et al.⁴

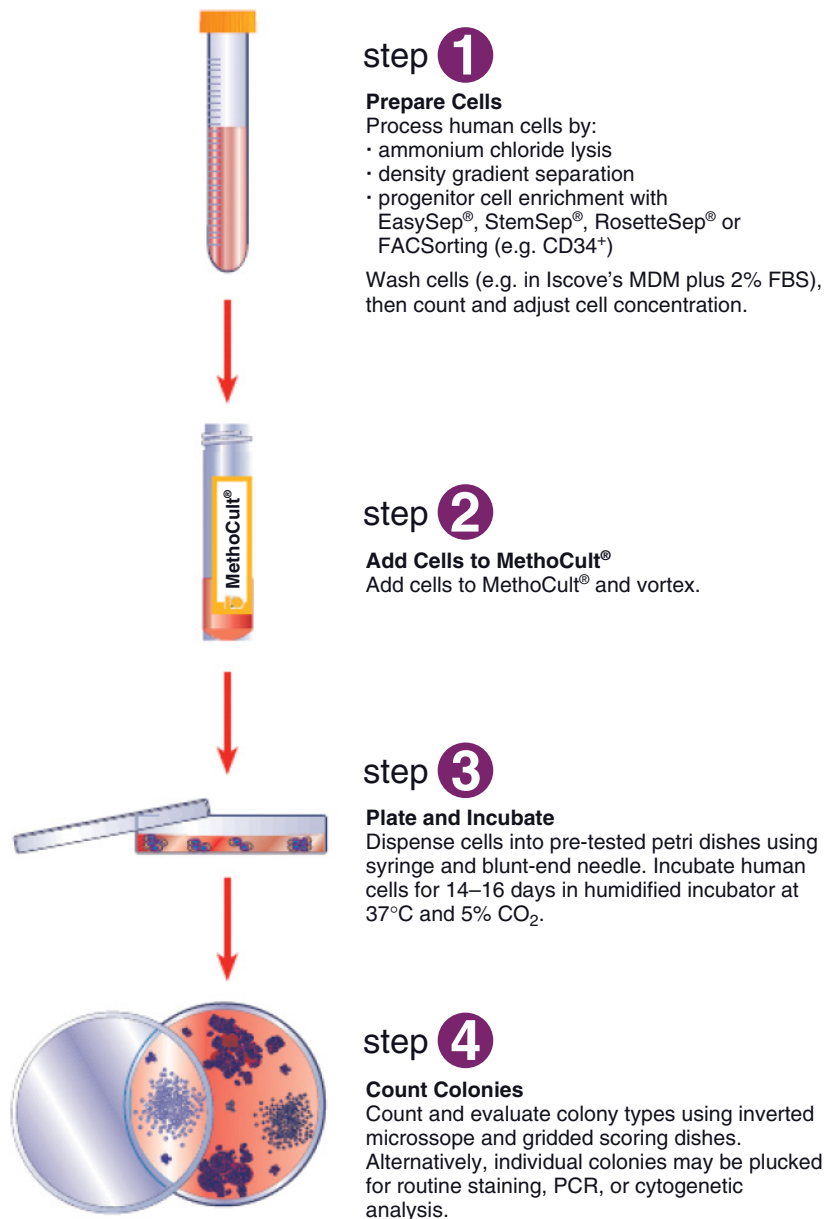
TYPES OF HEMATOPOIETIC CULTURE

Progenitor Assays

Progenitor assays are also known as clonogenic assays (Fig. 133.1). Hematopoietic mononuclear cells (derived from blood, bone marrow, fetal liver or spleen) are cultured in a semi-solid medium so that one dividing and differentiating cell remains in one location as a colony of variably differentiated cells. For example, an erythroid progenitor will produce a colony containing

varying numbers of rubriblasts, rubricytes, and metarubricytes. These colonies can be counted microscopically in the culture dishes and are identified by their particular morphology (Fig. 133.2). Mature erythroid colonies will appear orange-red because of their hemoglobin content. The semi-solid medium may consist of agar, methylcellulose, agarose, or plasma or fibrin clots. In general, larger colonies of a single type are derived from less differentiated progenitors (i.e. more proliferative potential). Also, mixed colonies (i.e. those with morphologies of more than one lineage) are derived from less differentiated progenitors (multipotential). The enumeration of progenitors from a mixture of HCs is usually done as a percentage of the original cells plated, rather than as an absolute number. Table 133.1 lists and describes the various progenitors derived from progenitor cell assays.

FIGURE 133.1 Schematic showing a typical progenitor assay. (Courtesy of STEMCELL Technologies.)



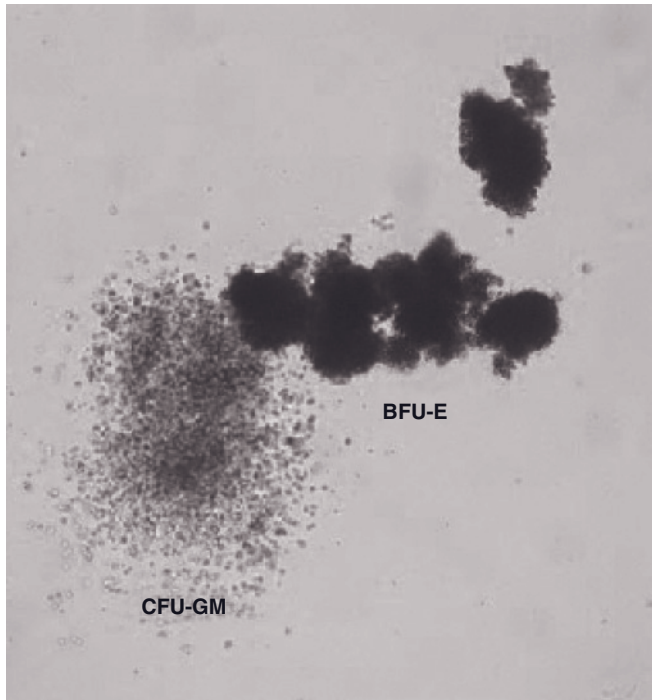


FIGURE 133.2 Photomicrograph of a CFU-GM colony and a BFU-E colony in a culture plate. (Courtesy of STEMCELL Technologies.)

TABLE 133.1 Hematopoietic Progenitor Cells and the Contents of the Subsequent Colonies

Progenitor	Differentiated Cell	Colony Size
CFU-GEMM	Granulocytes, macrophages, rubriblasts, megakaryocyte	>500 cells
BFU-E	Rubriblasts	>200 cells
CFU-GM	Granulocytes, macrophages	>20 cells
CFU-E	Rubriblasts	8–200 cells

Long Term Bone Marrow Culture (LTBMC)

This is a liquid culture system that consists of both an adherent and non-adherent cell population and recapitulates in vitro the in vivo relationships of bone marrow stromal and hematopoietic cells. Proliferation and differentiation of HCs occurs in liquid culture and production of progenitors can be assessed by culturing cells harvested from the liquid cultures in a subsequent progenitor assay. These cultures can be maintained for several months. The adherent layer consists of typical bone marrow stromal cell components, including fibroblasts, adipocytes, endothelial cells, and macrophages.¹¹ The cultures are initiated by a suspension of BMBCs and incubated until an appropriate stromal layer is formed. The cultures are re-infused with another BMBC suspension, and fed with media twice weekly. The cells of the microenvironment (stromal layer) produce growth factors and can support the growth of multipotent progenitor cells from which all mature

TABLE 133.2 Some Hematopoietic Cytokines and their Functions

Cytokine/ Growth Factor	Predominant Progenitors Stimulated	Other Cells Affected
M-CSF	Macrophages	Primitive progenitors
GM-CSF	Granulocytes/macrophages	Primitive progenitors
G-CSF	Granulocytes	Primitive progenitors
IL-3	Granulocytes, macrophages, megakaryocytes, eosinophils, mast cells	Erythroid (with EPO)
Stem cell factor	Primitive progenitors in synergy with other cytokines	N/A
EPO	CFU-E to erythroblasts	Megakaryocytes (synergistic)
TPO	Megakaryocyte colonies	Primitive progenitors

hematopoietic cells are generated. Lineage-committed cells will be released from the stroma as non-adherent cells and may then be enumerated using progenitor assays.⁶ LTBMC is primarily used to study the humoral and cellular mechanisms of hematopoiesis.³

Long Term Culture Initiating Cell (LTC-IC) Assay

An LTC-IC is a cell that can give rise to all of the progenitor cells described above over a 5–8 week period when cultured on an appropriate adherent “feeder cell” layer.¹⁰ The LTC-IC assay measures the frequency of these cells by performing progenitor assays after 5 weeks in culture and then inferring the number of LTC-ICs in the original culture. The majority of CFCs are in the adherent layer.² The frequency of LTC-ICs in human marrow is approximately 1 per 10^4 BMBC. LTC-ICs comprise 1 in 50–100 purified human CD34+ bone marrow cells.

GROWTH FACTORS USED IN HEMATOPOIETIC CULTURE

The growth of progenitors in semi-solid medium is not autonomous and therefore a variety of “growth factors” are required for their growth and differentiation. The reader is directed to an excellent review of hematopoietic cytokines by Metcalf.⁸ Each type of progenitor (e.g. CFU-GM, BFU-E, etc) responds to a certain set of growth factors, and thus many growth factors are named for the predominant lineage whose growth they stimulate. The growth factors were originally called “colony stimulating factors” (CSFs) because of this functional effect on progenitor assays. The CSFs were derived from media conditioned by immortalized cell lines or by mitogen stimulated primary cultures of mouse lung or

lymphocytes. Over a brief period of time, these growth factors were identified and cloned (Table 133.2). Although growth factors may have a predominant effect, they often synergize with other factors, or even affect the functions of mature hematopoietic cells. If growth factors are not added to the growth medium, many can be supplied using an adherent “feeder layer” of cells which produce them.

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Radiolabeling and Scintigraphic Imaging of Platelets and Leukocytes

DOUGLAS J. WEISS

Indications for Platelet and Neutrophil Scintigraphy
Platelet Scintigraphy with ^{111}In -Labeled Platelets
Alternative Techniques for Detection of Thrombi

Neutrophil Scintigraphy
Alternative Methods for Labeling Sites of
Inflammation

Acronyms and Abbreviations

^{18}F -FDG, fluorine-18-fluoro-2-deoxy-d-glucose; ^{111}In , indium-111 oxine; $^{99\text{m}}\text{Tc}$, technetium-99m hexamethylpropyleneamine oxime.

Platelet and neutrophil scintigraphic imaging studies have not been extensively used in veterinary medicine either in clinical medicine or in preclinical safety assessment studies. These techniques have been applied more widely in human medicine.^{1,5,9,13} Older techniques consisted of *ex vivo* radiolabeling of platelets, neutrophils, or mixed leukocytes followed by intravenous injection. Although these techniques have been reported to be effective, they do require a somewhat lengthy sterile isolation procedure. Several alternative methods have been reported in which radiolabeled compounds that bind to platelets or neutrophils at sites of thrombosis or inflammation are injected intravenously. Many of these involve administration of labeled monoclonal antibodies.^{5,12} These techniques have the advantage of considerably less pre-imaging preparation time but cannot be used for determination of platelet or neutrophil survival in the blood.

This chapter will discuss the indications for use of platelet or neutrophil scintigraphic imaging. Techniques for radiolabeling platelets and neutrophils and alternative approaches for detection of thrombi and sites of inflammation in the body will also be described.

INDICATIONS FOR PLATELET AND NEUTROPHIL SCINTIGRAPHY

Platelet scintigraphy has been primarily used for detection of thrombosis in human medicine.^{1,13} Because this is less of a problem in veterinary medicine, the technique has not been extensively applied. However, the technique has been applied to detection of platelet sequestration in laminar vessels in experimental equine

laminitis and in preclinical safety assessment studies.^{18,19} Many experimental compounds cause thrombocytopenia as an adverse effect. Platelet scintigraphy can be used to determine the effect of the compound on platelet survival in the blood, platelet distribution in the body, platelet recirculation after discontinuing the compound, and platelet deposition in thrombi.¹⁹ Platelet scintigraphy has been used to investigate the thrombocytopenia associated with administration of a glycoprotein IIb/IIIa peptide antagonist to dogs.¹⁹ Administration of the peptide resulted in a reduction in platelet lifespan and removal of platelets by the spleen. Platelet scintigraphy has also been used to investigate the role of platelets in equine laminitis.¹⁸ Near to the onset of lameness, radiolabeled platelets accumulate on the hoof wall of affected hooves. This is associated with decreased blood flow to affected hooves.

Neutrophil scintigraphy is largely used for identification and quantification of inflammatory processes.^{3,5,7} Occult inflammatory processes occur in all species but are a particular problem in horses.^{3,4,7,8} Most difficult to diagnose are intra-abdominal abscesses, ulcerative colitis, chronic pulmonary disease, and chronic bone and tendon inflammation that causes lameness. Neutrophil scintigraphy has been used to identify intra-abdominal abscesses, acute ulcerative colitis, and orthopedic infections in horses.^{4,7,8}

PLATELET SCINTIGRAPHY WITH ^{111}IN -LABELED PLATELETS

Platelets from a variety of species including horses, dogs, and rabbits have been labeled with indium-111 (^{111}In) oxine and successfully used for platelet scintigra-

phy based on the method originally described by Thakur et al.^{16,18,19} The volume of blood collected varies with the species. For horses, 250 mL of blood is collected whereas for dogs 50 mL of blood has been collected.^{3,7,19} Blood is aspirated directly into Aster's acid-citrate-dextrose solution (pH 4.5; 6 parts blood to 1 part acid-citrate-dextrose solution). Blood is maintained at room temperature throughout because cooling to 4°C permanently reduces platelet function. For horses, the blood is centrifuged at 150 × g for 15 minutes, whereas for dogs blood is centrifuged at 300 × g for 15 minutes. Platelet-rich plasma is placed in sterile tubes and centrifuged at 1,000 × g for 10 minutes. Thereafter, platelet-poor plasma is removed, and the platelet button is overlaid twice with 1–2 mL of modified Tyrode's buffer (pH 6.5). Platelets are resuspended in 4–6 mL of modified Tyrode's buffer. For horses approximately 1 mCi of ¹¹¹In oxine is added, whereas for dogs approximately 0.4 mCi of ¹¹¹In oxine is added.^{18,19} Total counts per minute in the mixture are determined by use of a gamma particle counting instrument. The sample is incubated for 10 minutes at 20–22°C and centrifuged at 1,200 × g for 10 minutes. The platelet pellet is overlaid twice with platelet-poor autologous plasma, and resuspended in autologous plasma. Total counts per minute in the sample are determined, and labeling efficiency is calculated. Labeled platelets are re-administered intravenously, usually within 2 hours after blood collection. For determination of platelet lifespan, the radioactivity in 1 mL of blood is determined at least once a day for 6 days, and results are corrected for background and physical decay and blood loss due to phlebotomy.^{16,19} Linear curves of percent platelet survival are plotted against time, and platelet half-life is determined. Some curves are linear whereas other curves are logarithmic. A weighted mean method can be used to compare platelet survival between plots that best fit linear versus logarithmic regression plots.¹⁹ Platelet survival can also be compared by determining the time for whole blood radioactivity to decrease from 100% to 50%.

Scintigraphic imaging is performed using a large field of view gamma camera. Because it is essential to keep the animal motionless for several minutes, sedation is helpful. Given a normal lifespan of 6–7 days, radioactivity is slowly cleared from the blood and accumulates primarily in the spleen within 1 week after injection. When using ¹¹¹In-labeled platelets to label thrombi, scans are usually done 6–24 hours after injecting the platelets for venous thrombi and 3–4 days for arterial thrombi.^{12,13}

ALTERNATIVE TECHNIQUES FOR DETECTION OF THROMBI

For clinical purposes a variety of other imaging techniques are replacing specific thrombus-labeling techniques. These include ultrasonography (Doppler), magnetic resonance imaging, and computerized tomography.¹⁰ However, specific thrombus labeling tech-

niques still have some clinical applications. Because ¹¹¹In-labeled platelet labeling techniques require up to 2 hours to process the platelets, several investigators have proposed less cumbersome imaging procedures to detect thrombi. These techniques include ^{99m}Tc-antifibrin, ^{99m}Tc-annexin V, ^{99m}Tc-anti-P-selectin F(ab)2, and ^{99m}Tc-platelet glycoprotein IIb/IIIa receptor antagonist.^{1,11,12,15} The details of these techniques will not be discussed here. Advantages of these procedures are a significant decrease in preparation time and a decrease in the time between injection and imaging. Imaging can usually be done within 1–6 hours after administration of the radioactive compound. Anti-P-selectin and other anti-platelet antibodies have been used to image thrombi in dogs.¹⁴

NEUTROPHIL SCINTIGRAPHY

Although neutrophil labeling can be used to determine neutrophil lifespan, its primary application has been for detecting sites of inflammation.^{3,4,7,8} Clinically the technique has primarily been adapted for use in humans and the horse but has also been applied to other domestic animals, including sheep.^{3,4,6,7,8} Neutrophils have been labeled with ^{99m}Tc-hexamethylpropyleneamine or ¹¹¹In-oxine. In horses, the technique can be performed quite simply by labeling mixed leukocytes after sedimentation of erythrocytes. Alternatively, pure populations of granulocytes can be isolated by use of continuous or discontinuous density gradient centrifugation. Approximately 250 mL of anticoagulated whole blood is collected from horses and erythrocytes are allowed to sediment for 20–30 minutes.⁷ Thereafter leukocyte-rich plasma is removed and centrifuged at 150 × g for 15 minutes.³ Plasma is removed and cells are resuspended in saline solution. Indium-111 oxine (300–400 μCi) is added and incubated for 15 minutes. The cell suspension is then washed in autologous platelet-poor plasma and resuspended in 15 mL of autologous platelet-poor plasma and reinjected intravenously. Images are collected using a large field-of-view gamma camera. Sites of inflammation are usually most visible at 24–48 hours after injection of leukocytes.^{3,7}

ALTERNATIVE METHODS FOR LABELING SITES OF INFLAMMATION

Alternative techniques for labeling sites of inflammation include ^{99m}Tc-labeled anti-granulocyte monoclonal antibodies and Fab' fragments, chemotactic cytokines, and fluorine-18-fluoro-2-deoxy-d-glucose (¹⁸F-FDG).^{5,17} As a glucose analog, ¹⁸F-FDG is rapidly taken up by sites of acute or chronic inflammation due to their increased glycolytic activity.^{2,5} Each of these compounds is injected intravenously. Scans for anti-granulocyte antibodies are usually obtained 2–24 hours after injection using a gamma camera. Scans for ¹⁸F-FDG are obtained within 1 hour after injection using positron emission tomographic imaging.^{2,5} Although some

differences of opinion still exist among physicians, ^{18}F -FDG appears to be preferred over anti-granulocyte antibodies.

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Automated Hematology Systems

ANDREAS MORITZ and MARTINA BECKER

Introduction and Historical Development
 General Principles of Hematology Instruments
 Electronic Impedance
 Light Scatter
 Centrifugal Analysis
 Hematology Instruments in Veterinary Medicine
 ADVIA 120/2120® (Siemens Healthcare
 Diagnostics)

Hemoglobin measurement
 Red blood cell and platelet measurement
 White blood cell measurement
 Reticulocyte analysis
 Cell-Dyn® 3500 (Abbott Laboratories)
 Sysmex XT-2000iV® (Sysmex Europe)
 In-House Hematology Instruments

Acronyms and Abbreviations

BASO, basophil; CHCM, corpuscular hemoglobin concentration mean; CHr, reticulocyte cellular hemoglobin content; DNA, Deoxyribonucleic acid; Hgb, hemoglobin; Hct, hematocrit; LI, lobularity index; LUC, large unstained cell; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MCVr, mean cellular volume reticulocyte; MN, mononuclear; MPC, mean platelet component concentration; MPO, myeloperoxidase; MPV, mean platelet volume; MPXI, mean peroxidase activity index; NRBC, nucleated red blood cell; PDW, platelet distribution width; PEROX, peroxidase; PLT, platelet; PM, platelet dry mass; PMN, polymorphonuclear cells; QBC, quantitative buffy-coat analysis; RBC V/HC cytogram, red blood cell volume/hemoglobin concentration cytogram; RBC, red blood cell; RDW, red cell distribution width; RETIC Scatter ABS, reticulocyte absorption scattergram; RNA, ribonucleic acid; SLS, sodium lauryl sulphate; SMA, sequential multiple analyzer; WBC, white blood cell; WIC, WBC impedance count; WOC, WBC optical count.

INTRODUCTION AND HISTORICAL DEVELOPMENT

Counting blood cells automatically began in the 1930s with a photoelectric method using darkfield optics.²⁹ Later, Langerkranz proposed the use of darkfield optics or light scatter to scan hemocytometer chambers to avoid human error.²² Instruments correlating the red blood cell (RBC) count with turbidity of cell suspensions did not gain wide acceptance because of inaccuracy and lack of practicability.³⁵ In the 1950s and 1960s, Coulter Electronics, Inc. and Technicon Instruments introduced analyzers able to determine one or several hematological parameters. The Coulter instrument was based on electrical impedance and the counting principle was patented, so no other manufacturer could produce such an instrument for 17 years.³⁵ The Technicon instrument was known as a sequential multiple analyzer (SMA) and utilized darkfield optical scanning to determine the RBC and white blood cell (WBC) counts, hemoglobin (Hgb) concentration, and hematocrit (Hct)

(SMA 4®, Technicon Instruments). The Coulter Model S® additionally calculated the mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV) according to well known formulas and was one of the pioneering “multiparameter” instruments. In subsequent years, more and more parameters, including platelet count, RBC distribution width (RDW), platelet distribution width (PDW) and mean platelet volume (MPV) were added to multiparameter analyzers and further technological developments, such as the combination of optical counting principle with cytochemical stains by Technicon Instruments, were introduced. In the mid 1970s Ortho Diagnostic Systems entered the multiparameter hematology instrument market and launched a cell-counting instrument which was based on laser light scatter and utilized the concept of hydrodynamic focusing. Pattern recognition systems were also developed in the 1970s, but were slow and did not improve precision of manual methods and are currently no longer manufactured.¹² Most large and in-house

hematology instruments are now capable of generating a WBC differential cell count. Methods for differentiating the WBCs vary between manufacturers, but include electrical impedance, laser light scatter, cytochemical stains, use of reagents capable of differentially lysing certain cell types, and combinations of these methods.

GENERAL PRINCIPLES OF HEMATOLOGY INSTRUMENTS

Electronic Impedance

Electronic impedance introduced by Coulter in the 1950s is still widely used in large and in-house hematology instruments. It is based on the fact that cells are poor conductors of electricity. If cells are suspended in an electrolyte solution and passed through a small aperture between two electrodes with continuous current, conductance will change. Change in conductance is sensed as a change in current, which is proportional to particle size. To count and size particles accurately thresholds have been developed. A threshold is a voltage limit with which a pulse is compared. Only pulses that exceed the threshold are sized and counted. Both a lower and an upper threshold can be defined and by manipulating the upper and lower thresholds, it is possible to produce a "window," which is a specific particle size range (Fig. 135.1).¹² In automated hematology instruments, these thresholds can be predefined (fixed) or floating. With floating thresholds, the software searches for a "valley" between two distributions and places a threshold at this location.¹⁸

In some hematology instruments (e.g. Sysmex E-Series®) radio frequency analysis has been combined with direct current volumetric sizing for simultaneous measurement of each WBC.¹² Radio frequency is high-frequency alternating current, which detects and sizes the cell on the basis of the overall density, including nuclear and granular density. In contrast, the direct current method sizes the entire cell, including nucleus and cytoplasm. Thus, the combination of direct current

and radio frequency offers information about cell size, size of the nucleus and volume fraction of the cytoplasm to the entire cell.⁴¹ Another possible way to improve the WBC differential cell counts is the combination of impedance technology with lysing reagents that strip away or shrink the cell's plasma membrane and cytoplasm, leaving "bare" nuclei.¹²

Light Scatter

Cells can be detected and counted as they pass through a beam of light instead of through an electrical field.¹² Although older instruments used mercury and halogen lamps, lasers are much more frequently used in modern hematology instruments. Cells passing through the beam of light cause diffraction, refraction and reflection and the scattered light is detected by appropriately placed photodetectors. With the darkfield optical method, the darkfield disc prevents light from hitting the photodetector when no cells are present. In contrast, with an absorbance channel, the photodiode receives maximum light when no cells are passing. Forward low-angle light scatter, forward high-angle light scatter, right angle scatter, and extinction offer information about cell volume, cell surface, lobularity, and granularity. Forward low-angle light scatter ($1-3^\circ$) correlates with cell volume; forward high-angle scatter ($4-9^\circ$) with granularity; right angle scatter (90°) with cell surface; and lobularity and extinction ($0-0.5^\circ$) is a measure for cell volume.

Flow cells are made of quartz rather than glass because quartz is transparent and does not bend light that passes through.¹² Flow cells are filled with sheath fluid. The sheath fluid prevents the flow cell from being coated by reagents or cell stroma. Sheath fluid also surrounds the sample stream and facilitates laminar flow and hydrodynamic focusing (see Chapter 137). Laminar flow ensures that all particles flow through the flow cell in parallel lines and that a second fluid containing the sample does not mix with the surrounding sheath fluid.¹² Hydrodynamic focusing is needed to assure that

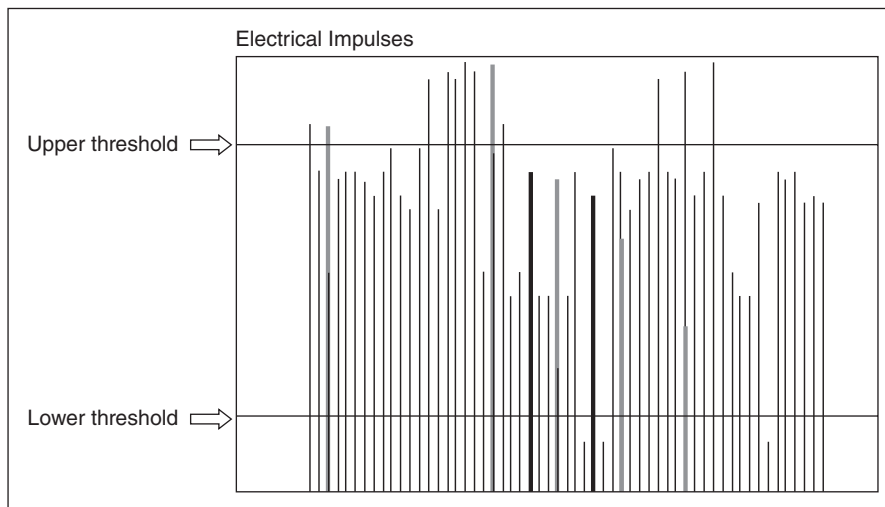


FIGURE 135.1 Diagram of electronic impedance. Number of impulses between the lower and upper threshold equals the number of particles passed through the aperture. The amplitude is proportional to the particle size.

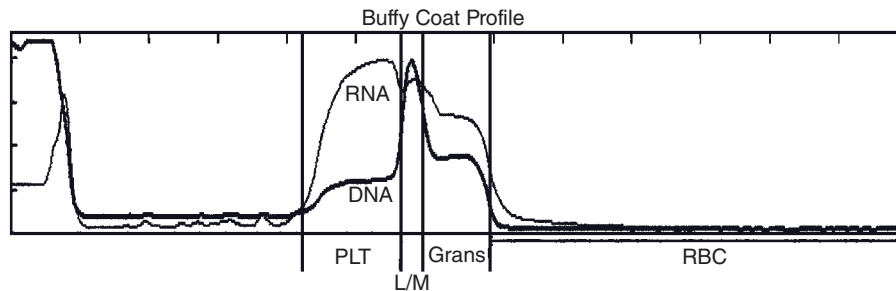


FIGURE 135.2 Buffy coat profile for a canine sample, analyzed on the QBC Vet Autoread®. Red blood cells do not fluoresce with acridine orange and thus the graph is near zero in the RBC area. The next layers are the granulocytes (Grans), which contain both RNA (thin line) and DNA (bold line). This is reflected by a hump in both lines. Below the platelets are the lymphocytes and monocytes (L/M). Because lymphocytes are smaller, they contain more DNA per volume. As a result a peak in the DNA line is visible. Platelets are at the top of the buffy coat and exhibit a DNA and RNA line. On the left edge of the graph, the free dye fluorescence and thus the DNA and RNA lines start high.

the cells pass individually through the flow cell. This is achieved by symmetrically decreasing the cross-sectional area of the fluidic channel in the flow cell and reducing the area in which the fluid is flowing. This results in faster-flowing central fluid with narrowing of the central sample stream.¹²

Centrifugal Analysis

With the centrifugal method, cells are not actually counted, but rather RBC and buffy coat layers are analyzed in centrifuged whole blood samples.¹² The method is based on the principle that different blood cells have different densities and that they sort into individual layers when spun in a microhematocrit tube. A cylindrical float (specific gravity is between that of plasma and RBCs), inserted into a capillary tube, expands the buffy coat layer. The inner surface of the capillary tube is coated with acridine orange, a fluorescent dye that stains a variety of different cellular components (DNA, RNA, lipoproteins, glycosamines): normal RBCs are not stained by acridine orange, granulocytes fluoresce orange-yellow, lymphocytes and monocytes brilliant green and platelets pale yellow. The fluorescence emitted by the cells in the tube is examined and values for WBC, PLT, MCHC, Hgb, Hct, granulocytes and lymphocytes/monocytes are reported; a characteristic buffy coat profile is generated (Fig. 135.2).

HEMATOLOGY INSTRUMENTS IN VETERINARY MEDICINE

The number of hematology analyzers available for veterinary medicine has increased steadily. In addition to large laboratory instruments, several smaller and affordable in-house instruments have been manufactured in recent years. These instruments are based on laser, impedance, or buffy coat technology and combinations of these methods. In this section we will discuss the most important analyzers.

ADVIA 120/2120® (Siemens Medical Solutions)

The ADVIA analyzers are flow-cytometry based systems, using light scatter, differential WBC lysis, and peroxidase and oxazine 750 staining to provide : (1) a complete blood cell count, (2) a WBC differential cell count, and (3) a reticulocyte count.²⁰ In contrast to the ADVIA 120®, the ADVIA 2120® uses a cyanide-free hemoglobin method, has a new user interface, and can be used for routine analysis of biological fluid samples in addition to blood samples.¹⁷

Hemoglobin Measurement

The ADVIA system has two methods of measuring Hgb: a modification of the manual cyanmethemoglobin method and a cell-based Hgb measurement. In the modified cyanmethemoglobin method, blood samples are mixed with the Hgb reagent in the Hgb reaction chamber. The Hgb chemical reaction consists of two steps: lysis of RBCs to release Hgb and formation of the reaction product, which is measured colorimetrically at a wavelength of 546 nm.⁶ The cell-based Hgb measurement determines the corpuscular Hgb concentration mean (CHCM). Comparable to the MCHC it provides a measurement of the average corpuscular Hgb concentration in the sample. However, the CHCM is directly measured based on a cell-by-cell analysis, while the MCHC is a calculated parameter ($\text{Hgb}/[\text{RBC} \times \text{MCV}] \times 1000$). Thus, interfering substances such as severe hemolysis or lipemia, which falsely elevate the Hgb and MCHC, will not affect the CHCM.⁶

The ADVIA 2120® analyzer can operate with a cyanide-free Hgb method. A comparison of this method to the modified cyanmethemoglobin method on the ADVIA 120® and 2120® showed a proportional bias, with overestimation of the Hgb values in dogs, cats, horses, and goats. However, the cyanide-free method was believed to be applicable in these species, provided the proportional bias is considered.^{4,5}

Red Blood Cell and Platelet Measurement

The RBC and platelet cytochemical reaction consists of two steps: RBCs and PLTs are isovolumetrically sphered with sodium dodecyl sulphate. The sphering eliminates shape as a variable. In the second step RBCs and PLTs are fixed with glutaraldehyde. A pair of low-angle light scatter (2–3°) and high-angle light scatter (5–15°) signals are used to analyze RBCs. Using the Mie theory of light scattering for homogeneous spheres, low-angle light scatter is converted into cell volume and high-angle light scatter is converted into hemoglobin concentration.⁶ Several histograms and cytograms are then used to determine and display RBC parameters: the RBC rate histogram shows the arrival rate of cells in the RBC/PLT channel. The RBC volume (RBC V) histogram displays the distribution of RBCs by volume. The RBC HC histogram shows the distribution of RBCs by Hgb concentration independent of cell volume, and the RBC CH histogram shows the distribution of RBCs by Hgb content. Additionally, the RBC Scatter cytogram (Fig. 135.3) is formed by plotting the high-angle light scatter (5–15°) on the *x*-axis and the low-angle light scatter (2–3°) on the *y*-axis. The RBC V/HC cytogram (Fig. 135.3) is a presentation of the RBC volume and Hgb concentration data intended for evaluating RBC morphology. The RBC Matrix then provides the cell counts and percentages for the corresponding nine regions on the RBC V/HC cytogram.⁶ Based on these RBC and Hgb measurements, several other parameters are determined (Table 135.1). Additionally, morphological flags such as microcytosis, macrocytosis, and anisocytosis, as well as hypochromasia, hyperchromasia, and anisochromasia are available. Thresholds can be defined for each species.²³ Figure 135.4 demonstrates the RBC V/HC cytograms for seven different species and reflects the different thresholds, developed for each species individually.

Platelets are analyzed with a two-dimensional method: the low-angle light scatter (2–3°) signal is amplified 30 times, and the high-angle light scatter (5–15°) signal is amplified 12 times. Comparable to

RBC analysis, the Mie theory of light scattering for homogeneous spheres is used to convert low-angle light scatter measurement into cell volume and high-angle scatter measurement into refractive index. Several histograms and cytograms are then utilized to determine and display platelet parameters:⁶ the PLT X histogram corresponds to the *x*-axis on the PLT scatter cytogram and displays the high-angle light scatter. The PLT Y histogram corresponds to the *y*-axis on the PLT Scatter cytogram and displays the low-angle scatter. The Platelet VOL histogram of the two-dimensional PLT analysis shows the distribution of cells by volume. The Platelet PM histogram displays the distribution of platelets by platelet dry mass (PM). The platelet PC histogram demonstrates the distribution of platelets according to refractive index (platelet component concentration [PC]). Furthermore, the PLT Scatter cyto-

TABLE 135.1 RBC Parameters, Determined by the ADVIA 120/2120®

Parameter	Explanation
Mean corpuscular volume (MCV)	Mean of RBC volume histogram
Red cell distribution width (RDW)	100 × (SD of RBC volume histogram/MCV)
Corpuscular hemoglobin concentration mean (CHCM)	Mean of RBC HC histogram
Hemoglobin concentration distribution width (HDW)	SD of RBC HC histogram
Cellular hemoglobin content (CH)	Mean of RBC CH histogram
Hematocrit (Hct)	(RBC × MCV)/10
Mean corpuscular hemoglobin (MCH)	(HGB/RBC) × 10
Mean corpuscular hemoglobin concentration (MCHC)	(HGB/[RBC × MCV]) × 1000
RBC fragments	Count of RBC fragments ^a
RBC ghosts	Count of RBC ghosts ^a

^aDetermined with two-dimensional platelet analysis and displayed in the PLT scatter cytogram.

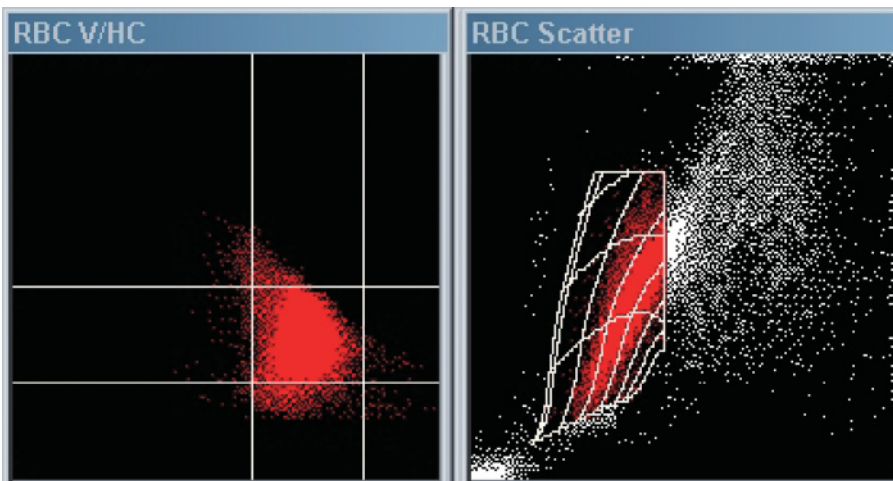


FIGURE 135.3 Red blood cell scatter cytogram and corresponding RBC V/HC cytogram of a normal dog. The RBC scatter cytogram is formed by plotting the high-angle light scatter (5–15°) along the *x*-axis and the low-angle light scatter (2–3°) on the *y*-axis. The RBC V/HC cytogram is a presentation of the RBC volume (*y* axis) and Hgb concentration (*x* axis) data intended for evaluating RBC morphology. The RBC V/HC cytogram is also called the Tic Tac Toe.

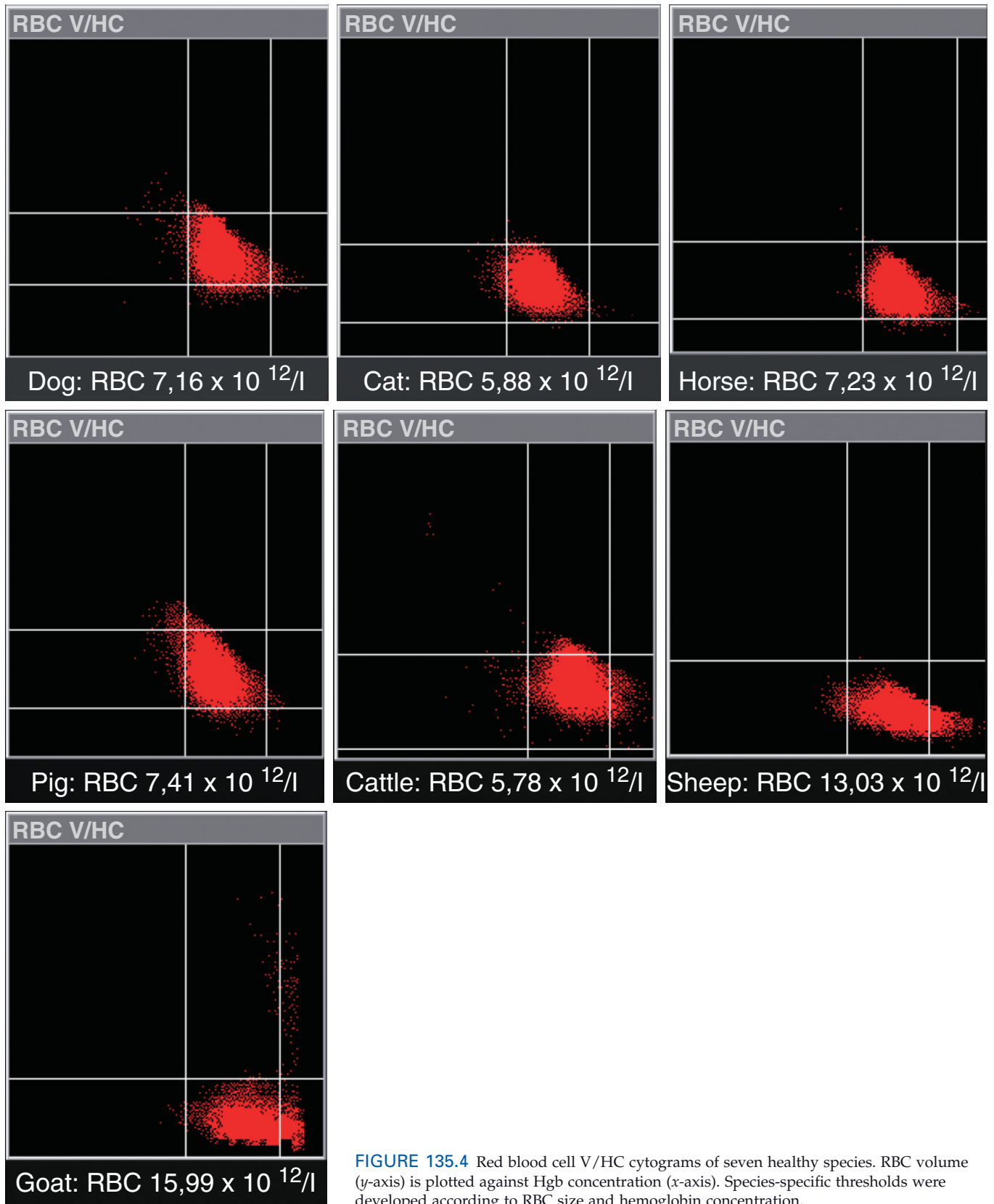


FIGURE 135.4 Red blood cell V/HC cytograms of seven healthy species. RBC volume (*y*-axis) is plotted against Hgb concentration (*x*-axis). Species-specific thresholds were developed according to RBC size and hemoglobin concentration.

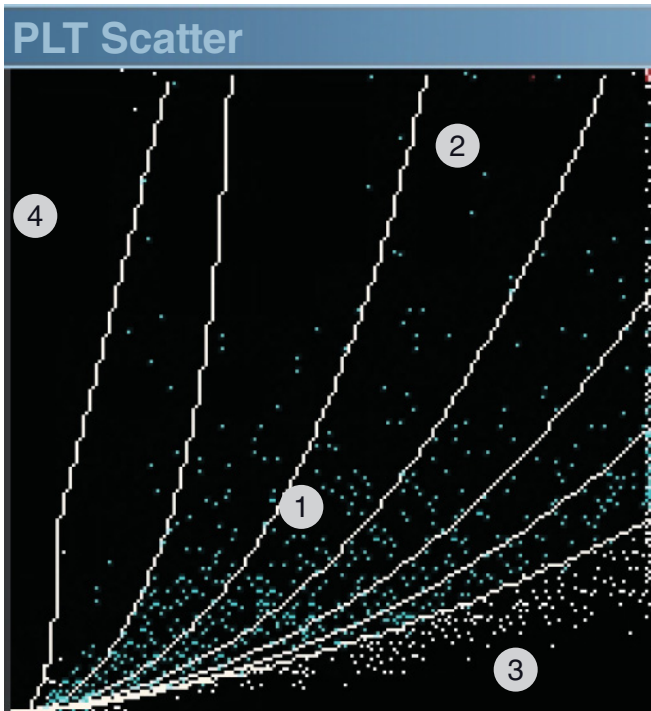


FIGURE 135.5 Platelet scatter cytogram of a healthy horse. The high-angle (5–15°) scatter is plotted on the x-axis, and the low-angle (2–3°) is plotted on the y-axis: 1, platelets; 2, large platelets; 3, RBC fragments; 4, RBC ghosts.

gram (Fig. 135.5) is the graphical representation of two light-scatter measurements: the high-angle (5–15°) is plotted on the x-axis, and low-angle (2–3°) is plotted on the y-axis. There is also a Platelet Volume/Refractive Index cytogram available, where the refractive index is plotted on the x-axis and platelet volume on the y-axis. Comparable to the RBC measurement, this two-dimensional platelet analysis offers many different parameters; some of them are only available for research purposes. The most important PLT parameters are shown in Table 135.2. Many of these parameters have been investigated for detection of activated platelets. A correlation has been found between an increase in P-selectin and a decrease in the mean platelet component concentration (MPC) in dogs with septic and non-septic inflammatory diseases.²⁴

White Blood Cell Measurement

White blood cells are counted and differentiated with two different methods: peroxidase staining and basophil/lobularity.

In the peroxidase cytochemical reaction, RBCs are lysed and 4-chloro-1-naphthol serves as a substrate that enables hydrogen peroxide to form a dark precipitate at sites of peroxidase activity in the granules of some WBCs.⁶ Neutrophils, eosinophils, and monocytes are stained based on their levels of peroxidase activity. Because lymphocytes, basophils, and large unstained cells contain no peroxidase, these cell types remain

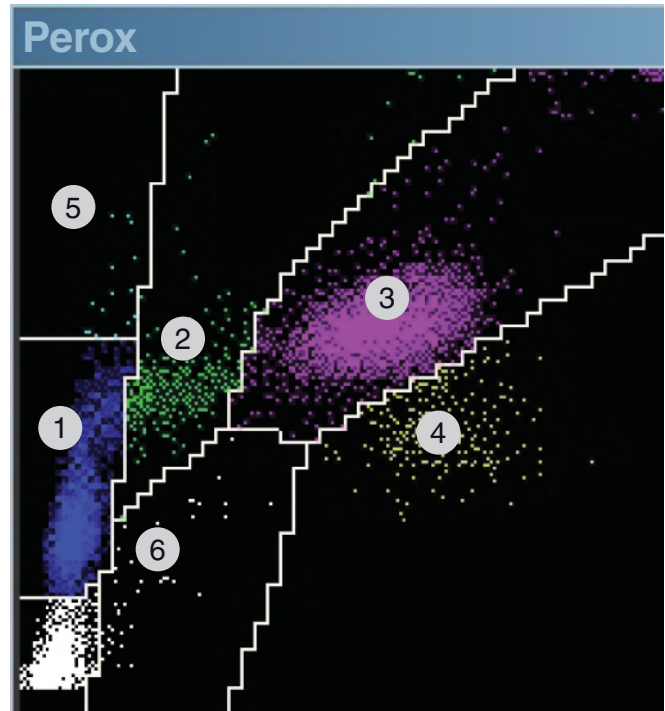


FIGURE 135.6 Peroxiside (PEROX) cytogram of a healthy dog. The cells absorb light in proportion to the amount of peroxidase stain present. This is represented on the x-axis. Cells size is represented on the y-axis (cells scatter light in proportion to their size). Distinct populations are formed when the light scatter and absorption data are plotted and cluster analysis then identifies each population: 1, lymphocytes and basophils; 2, monocytes; 3, neutrophils; 4, eosinophils (except cat); 5, large unstained cells; 6, platelet clumps.

TABLE 135.2 Platelet Parameters, Determined by the ADVIA 120/2120®

Parameter	Explanation
Mean platelet volume (MPV)	Mean of PLT volume histogram
Platelet volume distribution width (PDW)	100 × (SD of PLT volume histogram/MPV)
Mean platelet component concentration (MPC)	Mean of platelet PC histogram
Platelet component distribution width (PCDW)	SD of platelet PC histogram
Platelet crit (PCT)	(PLT × MPV)/10,000
Mean platelet dry mass (MPM)	Mean of platelet PM histogram
Platelet dry mass distribution width (PMDW)	SD of platelet PM histogram
Large PLT	Platelets with volumes greater than 20 fL

unstained. The results are graphically displayed in a PEROX cytogram (Figs. 135.6 and 135.7). The cells absorb light in proportion to the amount of peroxidase they contain. This is represented on the x-axis. Cells size

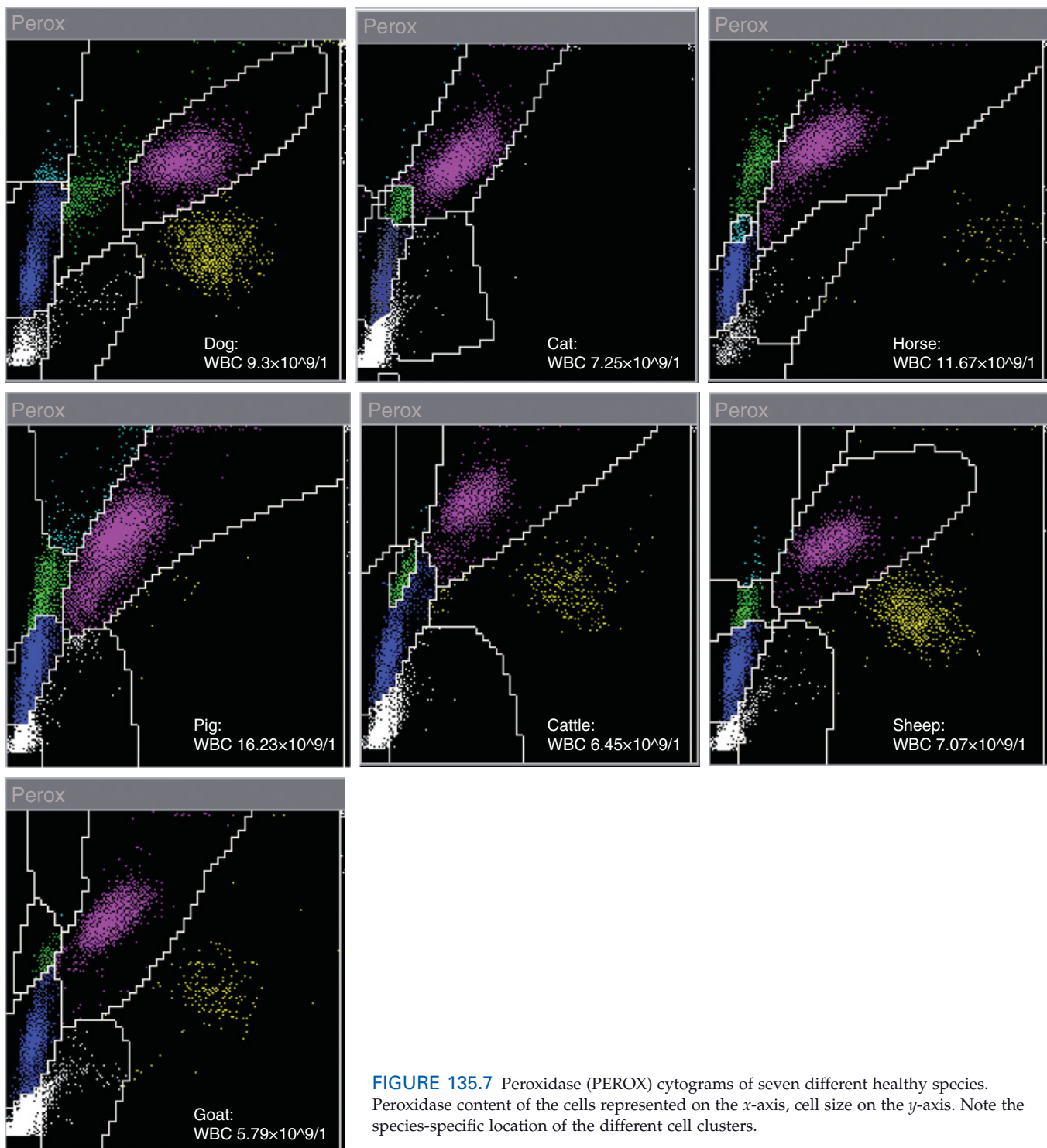


FIGURE 135.7 Peroxidase (PEROX) cytograms of seven different healthy species. Peroxidase content of the cells represented on the *x*-axis, cell size on the *y*-axis. Note the species-specific location of the different cell clusters.

is represented on the y -axis. Distinct populations are formed when the light scatter and absorption data are plotted and cluster analysis then identifies each population based on its position, area and density. These clusters are defined on a sample-by-sample basis. Besides a WBC count and five-part differential, including neutrophils, eosinophils, lymphocytes, monocytes and large unstained cells (LUCs), platelet clumps and mean peroxidase activity index (MPXI) are determined.

The MPXI was already available with the Bayer Technicon analyzer, the predecessor of the ADVIA 120. The system was used to identify persons suffering from myeloperoxidase deficiency (MPO).²¹ In veterinary medicine, dogs with pyometra were shown to have an increase in MPXI and the authors concluded that the parameter may be an indicator of bone marrow stimulation due to local inflammation or infection.²⁸ An increase in MPXI was also found in septicemic foals that were neutropenic.³¹ In contrast, normal MPXI values were found in septicemic foals that were not neutropenic.

Some species, (e.g. cat) have peroxidase-negative eosinophils, and eosinophils and neutrophils cannot be differentiated with the peroxidase method. Instead neutrophils and eosinophils are discriminated using the reticulocyte method (see later).

In the basophil/lobularity cytochemical reaction, RBCs and platelets are lysed and all WBCs except basophils are stripped of their cytoplasm by phthalic acid and a surfactant and by the increased temperature in the reaction chamber.⁶ Low-angle and high-angle light scatter are used to categorize the stripped WBCs as mononuclear (MN) or polymorphonuclear (PMN) cells based on the shape and complexity of their cell nuclei and to distinguish the intact basophils from the smaller cell nuclei. The results are displayed in the BASO cytogram (Fig. 135.8): the high-angle light scatter (nuclear configuration) is plotted on the x -axis, and the low-angle light scatter (cell size) is plotted on the y -axis. Cluster analysis is then applied to identify each population based on its position, area, and density. Beside a WBC and basophil count, the lobularity index (LI) is determined by this method. It reflects the valley between MN and PMN cells. In case of a left shift, the valley disappears and an instrument flag is displayed (only human software).

Both methods provide a total WBC count, which are compared as part of internal quality control procedure. In general, the WBC count obtained with the basophil/lobularity method is reported.

Reticulocyte Analysis

The cytochemical reaction for reticulocyte analysis consists of two steps: RBCs and platelets are isovolumetrically sphered and reticulocytes are differentially stained with Oxazine 750, according to their RNA content.⁶ Cells are then analyzed and differentiated with the low-angle and high-angle light scatter and absorption. Low-angle and high-angle light scatter are proportional to cell size and hemoglobin concentration, and absorption is proportional to RNA content. Results are graphically

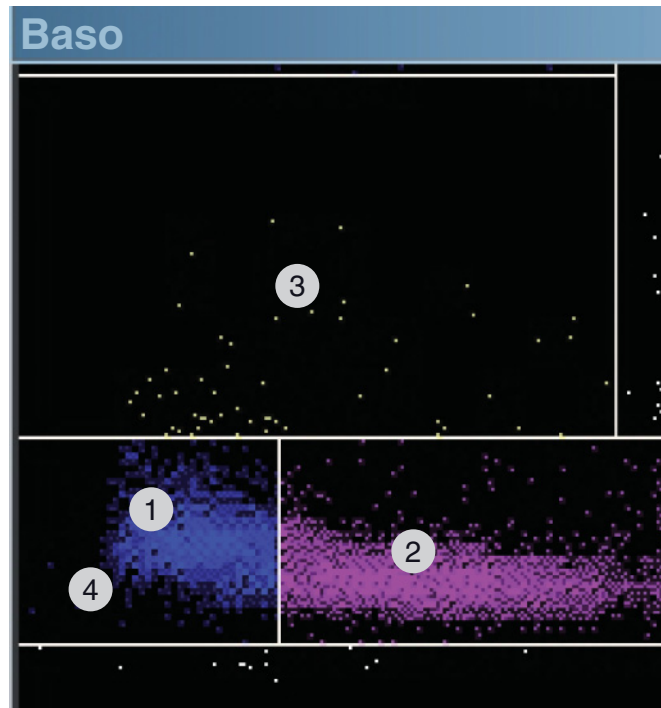


FIGURE 135.8 Basophil (BASO) cytogram of a healthy dog. The high-angle light scattergram (nuclear configuration) is plotted on the x -axis and the low-angle light scatter (cell size) is plotted on the y -axis. Cluster analysis is then applied to identify each population: 1, mononuclear WBCs (lymphocytes and monocytes); 2, polymorphonuclear WBCs (neutrophils and eosinophils); 3, basophils and lysis-resistant cells; 4: blast cell nuclei.

displayed in the RETIC Scatter ABS cytogram (Fig. 135.9): absorption (cell maturation) is plotted on the x -axis and high-angle light scatter (cell size) on the y -axis. Determination of cell maturation allows subclassification of reticulocytes into low, medium, and high absorption reticulocytes.

There is also a RETIC Scatter cytogram available, where the high-angle scatter is shown on the x -axis and the low-angle scatter on the y -axis. Mie theory is applied to transform the low-angle and high-angle scatter signals into volume and hemoglobin concentration values.⁶ In addition to the absolute reticulocyte count and the mentioned subpopulations, several other parameters are determined: the most important ones are the mean cell volume of reticulocyte population (MCVr) and reticulocyte cellular hemoglobin content (CHr). Both parameters have been used as markers of iron deficiency.^{10,14,34,45} MCVr and CHr hold promise as non-invasive, cost-effective measures of iron status in the dog.³⁴ Fry and Kirk, using a canine model of nutritional iron deficiency, showed that several reticulocyte parameters, including CHr and MCVr, were better indicators of developing and resolving iron deficiency than were conventional hematology or biochemical indices.¹⁴

Beside routine hematological analyses, the ADVIA 120® and ADVIA 2120® have also been used to evaluate different body fluids and bone marrow aspirates. The results of analyses of canine bone marrow samples

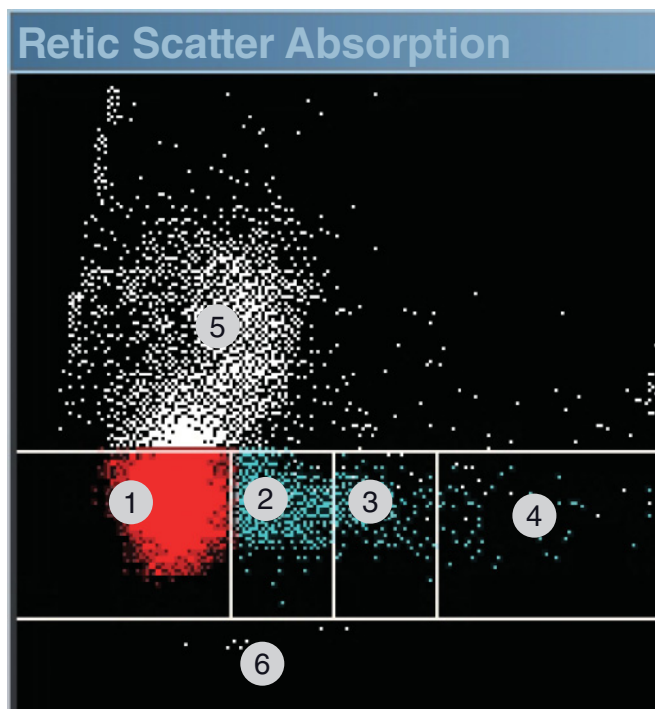


FIGURE 135.9 Reticulocyte (RETIC) scatter cytogram of a canine sample. Absorption (cell maturation) is plotted on the *x*-axis and high-angle light scatter (cell size) on the *y*-axis. Determination of cell maturation allows subclassification of reticulocytes into low, medium, and high absorption reticulocytes: 1, mature RBCs; 2, low absorption reticulocytes; 3, medium absorption reticulocytes; 4, high absorption reticulocytes; 5, coincidence events; 6, platelets.

showed that characteristic changes in the ADVIA 120® cytograms provided good preliminary information about the predominant cell population or the presence of haematopoietic neoplasia.² The instrument is also beneficial for the analysis of abdominal and pleural effusions and Bauer et al. showed that inflammatory effusions, monocytic proliferation and lymphoma could easily be detected. Carcinoma and mesothelial cells were classified as “mononuclear blasts.”³ Both analyzers have been evaluated for the analysis of cerebrospinal fluid.^{7,33} Promising results were obtained for RBC and total nucleated cell counts and the ADVIA 2120® was useful for classifying samples with significant pleocytosis.⁷

Cell-Dyn® 3500® (Abbott Laboratories)

Another large laboratory hematology instrument widely used in veterinary medicine is the Cell-Dyn® 3500. The analyzer determines a WBC optical count (WOC) and the WBC differential cell counts in an optical flow channel. Two different electric impedance channels are used for a WBC impedance count (WIC) and for RBC and platelet counts. Hemoglobin concentration is measured spectrophotometrically.¹

The following section highlights some important considerations about the WBC analysis on the Cell-

Dyn® 3500. As mentioned above, WBCs are analyzed in two separate channels and an algorithm is applied to compare the two counts and determine which value is reported. Appropriate warning messages are also displayed. In general, the WOC is reported as the WBC count.¹

For the WIC, RBCs are lysed and the reagent strips the cytoplasm from the WBCs. The WBC nuclei are then counted with an impedance method. Nucleated RBCs are included in the WIC.¹

In the optical channel, four different angles of scatter are measured: forward angle light scatter (1–3°), which correlates with the cell size, narrow-angle light scatter (7–11°), which correlates with cell complexity, ninety-degree light scatter (70–110°), which offers information about cell surface, lobularity, and ninety-degree depolarized light scatter, that correlates with certain types of cell granularity. These measurements allow a total WBC count, as well as a five-part differential cell count (i.e. neutrophils, eosinophils, basophils, lymphocytes, and monocytes) to be determined. Red blood cells are altered by the sheath reagent: hemoglobin diffuses out of the cell and water from the reagent diffuses into the cell. Thus, the RBC membrane remains intact, but the RBC now has the same refractive index as the sheath fluid, rendering it invisible to the laser.¹ The light scatter information is graphically presented in scatterplots: separation between MN and PMN cells is available through the ninety-degree/narrow angle scatterplot. The instrument uses dynamic thresholds to determine the best separation between the two populations. To further differentiate neutrophils and eosinophils, ninety-degree depolarized light scatter is plotted against the ninety-degree light scatter. The eosinophils scatter more 90° depolarized light than any other cells because of the unique nature of their granules and thus can be separated from neutrophils.¹ Finally, the mononuclear cells and basophils are separated by plotting forward-angle light scatter against narrow-angle light scatter. Basophils are included in the mononuclear clusters, because their granules are water soluble and dissolve in the sheath reagent.¹ Nucleated RBCs, unlysed RBCs, giant platelets and platelet clumps may also appear in this scattergram; however, they are excluded from the WBC count and WBC differential cell count. Additionally, several histograms are available (Fig. 135.10).

Available parameters and applicability of the Cell-Dyn® 3500 are much more limited in comparison to the ADVIA® analyzers. However, algorithmic analyses have been successfully applied to predict the ontogeny of leukemic cells in the blood of dogs and cats.¹³

Sysmex XT-2000iV® (Sysmex Europe)

The XT-2000iV® is a relatively new analyzer, combining aperture impedance and optical technologies. Red blood cells and platelets are counted in a dedicated channel using impedance combined with hydrodynamic focusing technology.³⁸ At the same time, the Hct is calculated via the RBC pulse height detection method:

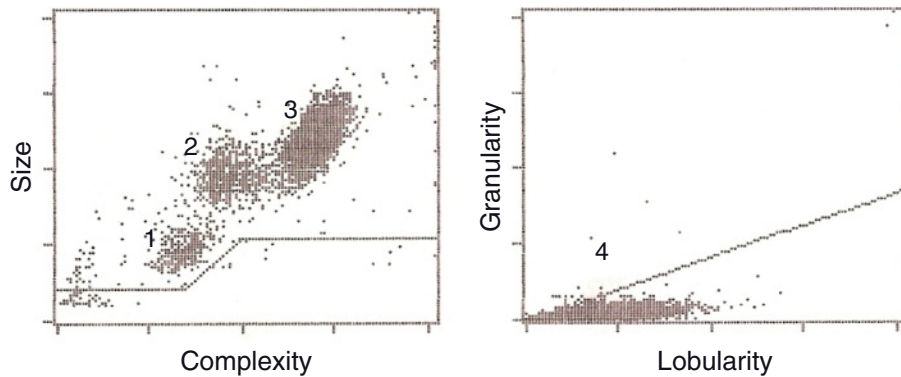


FIGURE 135.10 Cytograms for the Cell-Dyn® 3500 hematology analyzer (canine sample). On the left cytogram, cell size is plotted against complexity: 1, lymphocytes; 2, monocytes; 3, neutrophils. On the right cytogram, granularity is plotted against lobularity: eosinophils fall in the gate marked with number 4.

the intensity of the electronic pulse from each analyzed RBC is proportional to the cell volume. The analyzed pulses and the resulting RBC volumes of defined sample volume are accumulated to directly result in the Hct value.^{38,39} Instead of the more commonly used cyanmethemoglobin method, the sodium lauryl sulphate (SLS) method is applied to determine Hgb concentration: the anionic detergent forms a complex with Hgb, which is spectrophotometrically measured at 555 nm.

To determine an absolute reticulocyte count and to allow analysis of stages of maturity, the analyzer applies fluorescent dye and diode laser technologies.³⁶ The results are displayed in the RET Scattergram (see Chapter 136), where the *x*-axis represents the intensity of the lateral fluorescent light and the *y*-axis the intensity of the forward scattered light (Fig. 135.11).³⁹ The same principle is used to measure platelets. It has been shown that with this technology, the instrument is capable of reliably detecting large as well as immature platelets.²⁵

Forward-scattered light, lateral-scattered light and lateral-fluorescent light are used to determine the WBC count and WBC differential cell count. The results are displayed in a four differential scattergram, where the *x*-axis represents the intensity of the lateral-scattered light and the *y*-axis represents the intensity of the lateral-fluorescent light (Fig. 135.11).³⁷ In a second scattergram (WBC/BASO), lateral-scattered light is displayed on the *x*-axis, with forward-scattered light on the *y*-axis (Fig. 135.11).³⁷ In contrast to the other large laboratory hematology instruments, manual re-gating is possible with the Sysmex XT® analyzer. To the authors' knowledge, results of comparison studies concerning the accuracy of the Sysmex Analyzer are available only in abstract form. Our experience indicates that the system is relatively easy to use, and gives reliable results. Further studies are needed to determine the usefulness of the manual re-gating feature for WBCs. One study reports that the instrument can be used to determine total nucleated cell counts on body fluids of dogs and cats.³⁰

In-House Hematology Instruments

The point-of-care hematology instrument market is currently in flux, with frequent introductions of new instruments and enhancements. The decision about the type of system to be used depends not only on instrument accuracy and precision, but on other factors including costs, maintenance, ease of use, desired parameters, and the number of samples to be analyzed. The majority of the instruments use impedance technology to determine WBC, RBC, and platelet counts and parameters. A few instruments are available that apply laser technology to determine cell counts or at least the WBC differential cell count. Buffy-coat analysis is still used in one instrument (IDEXX VetAutoRead™, IDEXX Laboratories). Available parameters also vary between the different systems: two-, three- or five-part differential WBC counts and the ability to measure absolute reticulocyte counts are the most important differences between the analyzers.

Several studies concerning the accuracy of in-house instruments have been published.^{8,9,11,15,16,19,26,27,32,40,42-44} Comparing the results of the different investigations is difficult, because various reference/comparative methods, as well as different statistical methods have been used. Furthermore, many evaluations are only available in abstract form. In general, the point-of-care analyzers appear to perform well in comparison to the different reference or comparative method.⁸ There are some limitations, especially for the WBC differential cell counts and platelet counts. However, automated differential WBC counts, in a clinical setting, need to be verified no matter which automated instrument is used. Thus, with the currently available in-house instruments, accuracy seems not to be a major factor in selecting an instrument. Instead, as mentioned above, costs, maintenance, ease of use, desired parameters, and number of samples to be analyzed are more important considerations.

Besides assuring the accuracy of instruments, manufacturers must train users appropriately concerning

FIGURE 135.11 Routinely available data report of the Sysmex XT-2000iV® (canine sample). On the left, the numerical data and on the right the graphical report including cytograms: Diff, WBC/Baso, Ret, PLT-O, Ret-Ext and histograms: RBC and PLT.

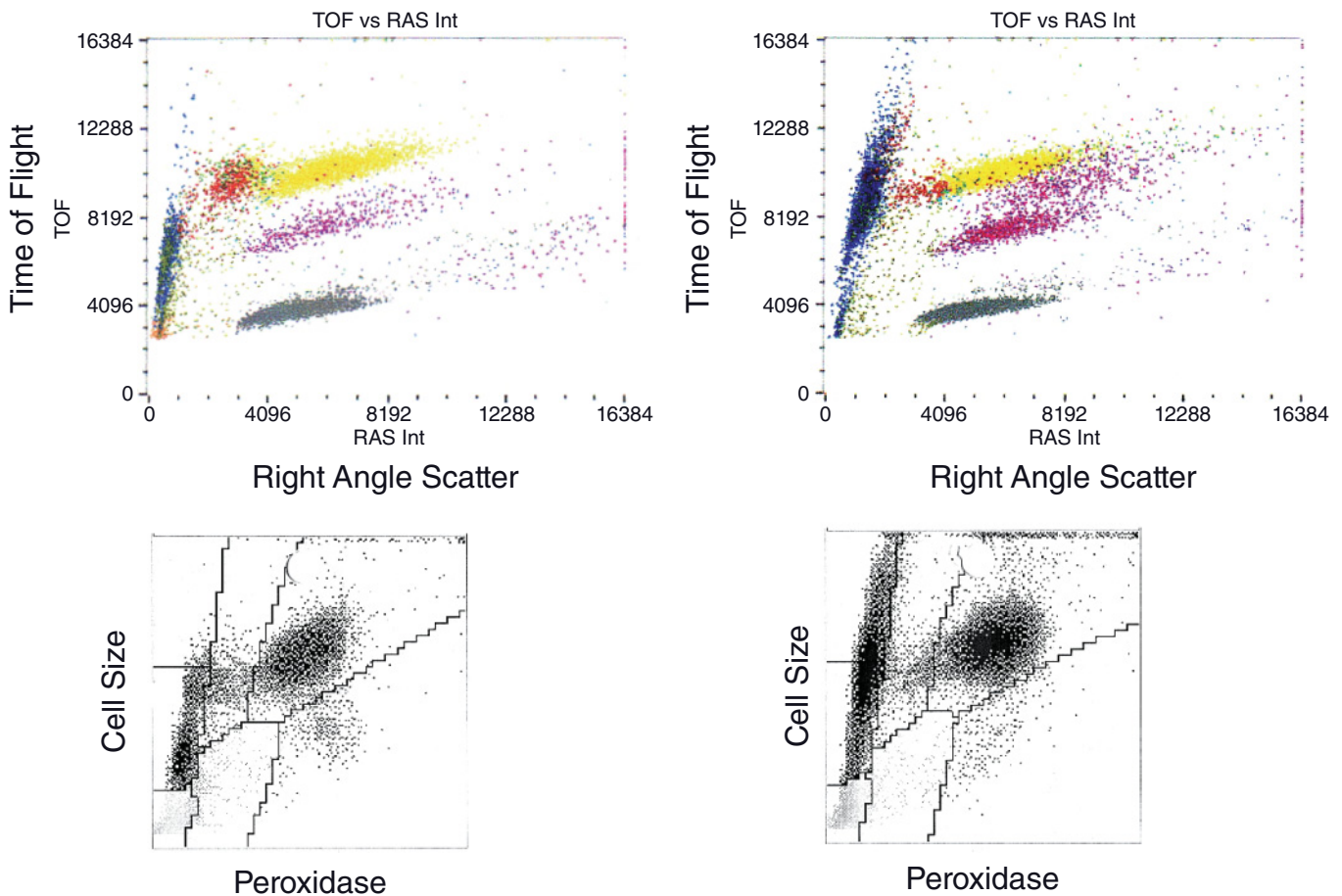
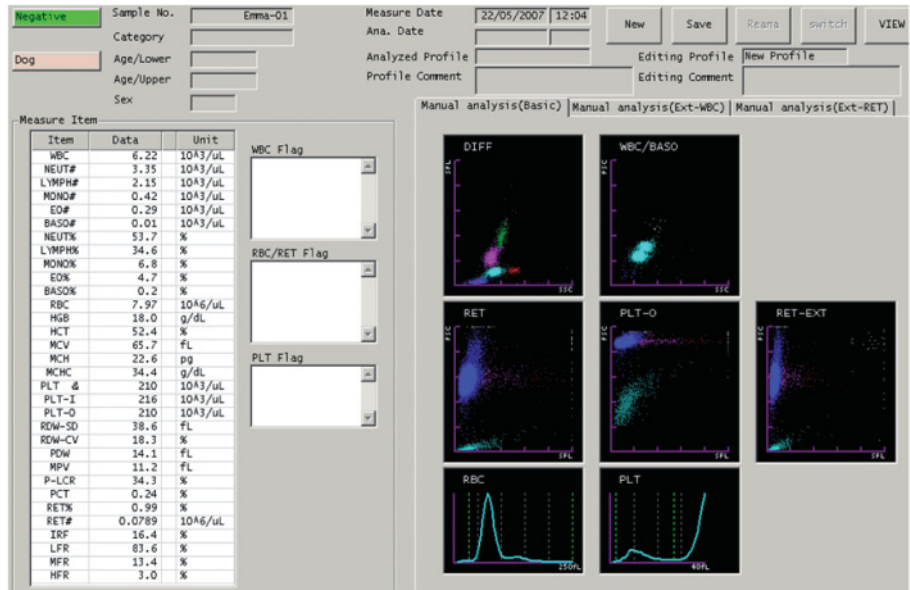


FIGURE 135.12 Cytograms of the LaserCyte® and ADVIA 120® for a hematological unremarkable sample (left) and a sample with a significant number of circulating lymphoblasts (right). The two scattergrams on the top are generated by the LaserCyte® and time of flight is plotted against right angle scatter: the blue population represents the lymphocytes. The two PEROX cytograms on the bottom are of the same samples, analyzed with the ADVIA 120®. The LaserCyte® scattergrams of the sample with lymphoblasts clearly reveals that the lymphocyte population is enlarged with numerous events scattering upwards. This is comparable to the PEROX scattergram of the ADVIA 120® with events spreading into the large unstained cells (LUC) gate.

operation, maintenance, quality-control procedures, troubleshooting, and most importantly, result verification. Result verification can be achieved in different ways: flagging of inaccurate results, assessment of graphical reports, and blood film evaluation. All hematology analyzers have the capacity to display error messages. The number of error messages and explanations provided by the manufacturer about the underlying causes for showing these messages vary greatly. Our own investigations revealed that up to 89% of test results are marked with an error message.⁸ Usefulness of these messages is very limited, because many accurate results are marked with an error message and, in contrast, many samples with inaccurate results are not detected.⁸ Thus, using error messages for result verification is currently not recommended. The impedance-based in-house instruments typically display histograms for WBCs, RBCs and platelets. A notable exception is the LaserCyte® (IDEXX Laboratories). This is a purely laser-based instrument, which also generates several scattergrams and histograms. Preliminary experience is promising concerning the usefulness of these scattergrams for verification of the WBC differential cell counts. Figure 135.12 shows the ADVIA 120® and LaserCyte® scattergrams of a normal canine blood sample and a sample with significant numbers of medium-sized lymphoblasts. In the displayed scattergrams of the LaserCyte®, time of flight is plotted against right-angle scatter and the blue population represents the lymphocytes. Comparing the scattergrams of the normal canine sample and the sample with circulating lymphoblasts, the lymphocyte population is enlarged with numerous events scattering upwards. This is comparable to the PEROX scattergram of the ADVIA 120® with events spreading into the large unstained cells (LUC) gate. However, prospective studies about sensitivity and specificity of scattergrams and histograms to recognize inaccurate results, as well as morphological changes (e.g. left shift, toxic changes, RBC poikilocytosis) are currently lacking. Therefore, blood smear evaluation remains essential for result verification and is currently needed for every sample analyzed on an in-house instrument.

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Reticulocyte and Heinz Body Staining and Enumeration

HAROLD TVEDTEN and ANDREAS MORITZ

Reticulocyte Staining and Counting

- Manual Methods
- Automated Methods
- Reporting Methods
- Other Indicators of Reticulocyte Status
- Reticulocyte Interpretation (see Chapter 24)

Heinz Body Staining and Counting

- Manual Methods
- Species Variations
- Interpretation of Heinz Bodies (see Chapters 24 and 36)

Acronyms and Abbreviations

Advia, Advia® 2120, 120 or 2120I; AO, acridine orange; BCB, brilliant cresyl blue; CH retic, hemoglobin concentration reticulocyte; CHr, cell hemoglobin of reticulocytes; EDTA, ethylenediaminetetraacetic acid; LaserCyte, IDEXX Laboratories LaserCyte® hematology analyzer; MCV retic, mean corpuscular volume reticulocyte; mRNA, messenger ribonucleic acid; NMB, new methylene blue; RBC, red blood cell; Sysmex, Sysmex XT® 2000 iV; TO, thiazole orange.

This chapter describes technical aspects of enumeration of reticulocytes and Heinz bodies. Interpretation of results is described in Chapter 24 and other chapters. Evaluation of reticulocytes and Heinz bodies occurs when blood is stained with a supravital dye, that demonstrates ribosomal material in reticulocytes and precipitated hemoglobin in Heinz bodies. Manual staining and counting of reticulocytes may be performed even in small clinics but is time consuming and subjective. Automated hematology instrument enumeration of reticulocytes is more precise and rapid and preferred for laboratories with appropriate instrumentation. Reticulocytes vary in appearance and number in various species of animals. Feline punctate reticulocytes have too little RNA to be detected by currently available automated veterinary hematology systems (i.e. Advia 2120®, Sysmex XT 2000 iV®) and must be counted manually on stained blood smears. Birds and reptiles have nucleated erythrocytes and variable numbers of ribosomes which can confuse identification and enumeration.

Heinz body evaluation is usually based on manual counting of the percentage of red blood cells (RBCs) that contain Heinz bodies in stained smears. The clinically important measurement of total mass of precipi-

tated hemoglobin (i.e. Heinz body material) does not consistently correlate with the percentage of RBCs containing Heinz bodies. The size and number of Heinz bodies should be considered in interpretation of severity of the change.

RETICULOCYTE STAINING AND COUNTING

Reticulocytes are the stage in the maturation of erythroid cells between the metarubricytes and mature RBCs. The basic principle of different methods identifying reticulocytes is to stain mRNA in ribosomes of reticulocytes so that they can be differentiated from mature RBCs and nucleated RBCs. In automated systems, the reticulocytes may be further divided into two or three subgroups based on the relative amount of mRNA they contain. Those with most mRNA are considered younger reticulocytes.

Based on the system of detection, a definition is required of how much mRNA or number of dots of ribosomal material is needed to classified an RBC as a reticulocyte. Punctate reticulocytes are frequently not detected by automated hematology systems. Even with manual reticulocyte counting, erythroid cells with only

1–2 dots of ribosomal material are not classified as reticulocytes.

Specimens for reticulocyte quantification are anticoagulated blood, usually in ethylenediaminetetraacetic acid (EDTA). Specimens are suitable if stored at room temperature for up to 48 hours and likely up to 4 days at 4°C. Specimens should be free of clots, hemolysis or other gross defects. Sample-induced errors in reticulocyte counting include Howell-Jolly bodies, basophilic stippling, blood parasites, large platelets with abundant RNA, and factors that affect determination of the total RBC count.

Manual Methods

RNA is a polyanion and can be stained with many cationic dyes including brilliant cresyl blue (BCB), new methylene blue (NMB), acridine orange (AO), thiazole orange (TO), azure B, auramine O, oxazine 750 and pyronine Y. The two stains used most frequently for manual counting of reticulocytes in blood smears are new methylene blue (1% NMB in saline plus 1.6% potassium oxalate) and brilliant cresyl blue (1% in saline). Equal amounts of blood and stain are mixed and allowed to stand at room temperature: 10 minutes for NMB and 15–30 minutes for BCB. One may increase the proportion of blood if the animal is very anemic. The mixture is remixed and blood smears are made. Counterstaining with a Romanowsky's stain (e.g. Wright-Giemsa) gives a darker reticulum with BCB. For birds, mix 3 drops NMB to 1 drop EDTA blood and allow to stand at room temperature for 10 minutes before making the smear.

Microscopic evaluation of 1000 stained non-nucleated erythroid cells is used to determine the percentage of reticulocytes. That percentage multiplied by the absolute erythrocyte count gives the absolute reticulocyte count per given volume (microliter or liter). Other granular material may be present and should be recognized. Heinz bodies stain a lighter blue than the more purple reticulocyte granules.

Reticulocytes are defined as RBCs that contain more than 2 dots of ribosomal material. Identification of the division between feline aggregate and punctate reticulocytes is more difficult and subjective (Fig. 136.1). Tvedten defines aggregate reticulocytes as having more than 15 dots or as RBC having one or more aggregates of dots. Reticulocytes counted in birds should be the ring form reticulocyte, in which aggregates of ribosomes are arranged in a ring around at least half of the nucleus. These correlate best with polychromasia in birds.⁴

Automated Methods

Automated systems have better precision and accuracy than manual methods because much larger numbers of cells are enumerated, inter-observer variation is avoided and variation in staining quality such as stain precipitates and staining intensity is minimized. Time needed

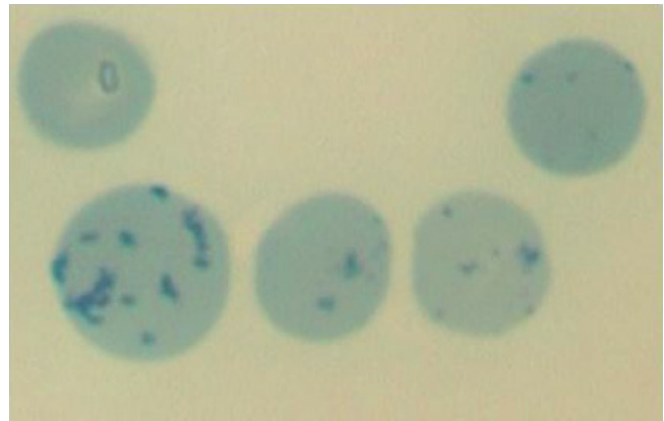


FIGURE 136.1 Feline reticulocytes illustrated are three punctate reticulocytes on the right, an aggregate reticulocyte on the lower left and an erythrocyte (with an artifact) on the upper left. The upper right punctate reticulocyte has three dots (ribosomal material) and it therefore just makes the definition of a reticulocyte.

to perform the test is greatly decreased; therefore reticulocyte evaluation becomes routinely available. The coefficient of variation for manual counting of reticulocytes may be 8–23% while for flow cytometric counting it may be less than 3%; however, stains and detection methods may result in variability in sensitivity and specificity.⁹

Automated veterinary instruments, designed for reference laboratories and validated to enumerate canine, feline, and equine reticulocytes, include the Sysmex® and the Advia®.⁵ Two in-clinic hematology instruments (IDEXX LaserCyte® and Oxford Science ForCyte®) have been evaluated for reticulocyte counting in dogs and they had fair correlation coefficients compared with the Advia®, and moderate random error for canine reticulocyte counting.¹ The LaserCyte® compared well with the Advia® for feline reticulocyte counts.

The Sysmex XT 2000 iV® uses fluorescence flow cytometry with a semiconductor laser for enumerating reticulocytes. Cells are stained with polymethime, a fluorescent stain, which is optimized to stain RNA.

Other stains, such as TO, AO, and auramine O, are used for flow cytometry.² Perkins and Grindem⁹ showed that of these three dyes, TO best identified distinct peaks corresponding to aggregate and punctate reticulocytes from cats, while using a dedicated reticulocyte cytometer, the Sysmex R-1000®, and a software package, Retic-count® (Becton-Dickinson Immunocytometry Systems, Sana Jose, CA). Thiazole orange was brighter than auramine O or AO, but required longer incubation times, and the commercially prepared reagent was expensive. Punctate reticulocytes appeared as a widened RBC peak or a shoulder off the mature peak. Aggregate reticulocytes formed a separate peak. Indistinct separation of the punctate reticulocytes required a standardized protocol and use of template histogram regions. Percentages of automated counts matched fairly well manual count results, with a cor-

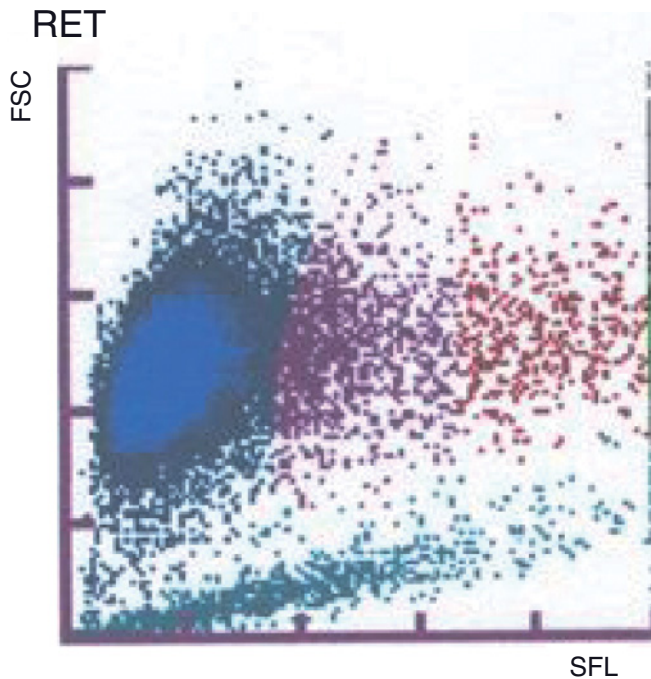


FIGURE 136.2 Reticulocyte dot plot from a cat with Sysmex XT 2000 iV® graphics. Example of marked reticulocytosis (10.3%). Mature RBCs are blue. Reticulocytes are purple and red and extend to the right of the RBCs. Platelets form the linear group of blue-green dots at the bottom. FSC, forward scatter; SFL, side fluorescence which is associated with RNA content.

relation coefficient of 0.72 with aggregate and 0.58 with manual punctate counts.⁹

The automated reticulocyte analysis of the Advia® uses a reagent solution formulated to maintain pH 7.4 as a preferred value, with an osmolality preferably about 292 ± 5 mOsm. At these pH and osmolality ranges, the concentration of the cationic dye, oxazine 750, required for RNA staining is preferably about $9.0 \mu\text{g}/\text{mL}$ to about $10.5 \mu\text{g}/\text{mL}$. The buffer enhanced penetration results in the dye staining RNA in the reticulocytes in about 20–30 seconds or less. The concentration of dye in the reagent of the method minimizes non-reticulocyte staining of mature RBCs which leads to a good signal separation from the background noise.¹⁴

Sysmex® canine reticulocyte counts had high correlation ($r = 0.90$) with manual counts.⁵ Sysmex® reticulocyte counts in cats (Fig. 136.2) correlated well with manual aggregate reticulocyte counts ($r = 0.86$) but not with manual punctate reticulocyte counts ($r = 0.50$). A mean positive bias of the Sysmex® reticulocyte count over the manual aggregate count was about 6% (range -2% to $+19\%$). This suggested that some punctate reticulocyte were included with the Sysmex® automated reticulocyte count. Equine reticulocytes are detected by the Sysmex® but too few were seen in manual reticulocyte counts to validate any accuracy. The highest absolute reticulocyte counts were seen in horses with highest RBC counts and in foals.⁵

The Advia 2120® automated reticulocyte counts (Fig. 136.3) had excellent correlation to manual reticulocyte counts in 32 dogs ($r = 0.94$, slope 0.98) and feline aggregate reticulocyte counts in 33 cats ($r = 0.94$, slope 1.16) (unpublished data). However, there was poor correlation with feline punctate reticulocytes. Equine reticulocytes are detected by the Advia®.¹²

The LaserCyte® uses NMB to stain reticulocytes. Reticulocytes are then differentiated from RBCs by different extinction and laser light scatter. The LaserCyte® was compared to Advia® and manual reticulocyte counting in 250 anemic dogs in which two-thirds had a regenerative anemia defined by more than $60 \times 10^3/\mu\text{L}$ reticulocytes on manual counts.¹³ Weissert et al. found a good correlation ($r = 0.66$ – 0.72) but a negative bias combined with a proportional error for the LaserCyte® absolute reticulocyte count compared to the manual and Advia® methods. Thus they recommended instrument specific cut-off values for the LaserCyte® to separate nonregenerative from regenerative anemia which have reticulocytes of $<35 \times 10^3/\mu\text{L}$ or $>75 \times 10^3/\mu\text{L}$, respectively.¹³

Reporting Methods

Reticulocytes are reported both as a percentage of non-nucleated RBCs and absolute number per liter or microliter. The absolute number of reticulocytes is recommended for interpretation. However, the total RBC count is needed to calculate the absolute count ($\text{RBC} \times \text{percent reticulocytes}$; see Chapter 22).

Other Indicators of Reticulocyte Status

The Advia® identifies the volume and hemoglobin concentration of each individual RBC analyzed and keeps track of which cells are reticulocytes (Fig. 136.4). Thus the instrument can report reticulocyte size (MCV retic) and hemoglobin concentration (CH retic) by summation of individual results. CH retic and MCV retic have been shown to be sensitive indicators of iron deficiency and response to therapy in dogs (Fig. 136.4).³

Note in Figure 136.4 that the reticulocyte cluster in RTC SCAT ABS in the upper left is lower than the cluster in Figure 136.3 as the reticulocytes in iron deficiency are relatively smaller (more microcytic) than normal reticulocytes. Reticulocytes in iron deficiency are still relatively macrocytic and hypochromic compared to the patient's own erythrocytes (Fig 136.4). Note the blue colored reticulocytes in the RTC volume and RTC HC histograms at the lower right are more macrocytic and hypochromic than the RBCs indicated by the red peak. Reticulocyte volume (MCVr) was 65.5 fL and cell hemoglobin of reticulocytes (CHr) was 15.4 pg. Reticulocyte indices may be monitored for response to treatment. For example, when assessing values after experimental iron replacement in iron deficient dogs, the MCVr increased from 65 fL before iron treatment to 82 fL after 11–12 days of iron supplementation, and CHr increased from about 16 pg up to 22 pg.³

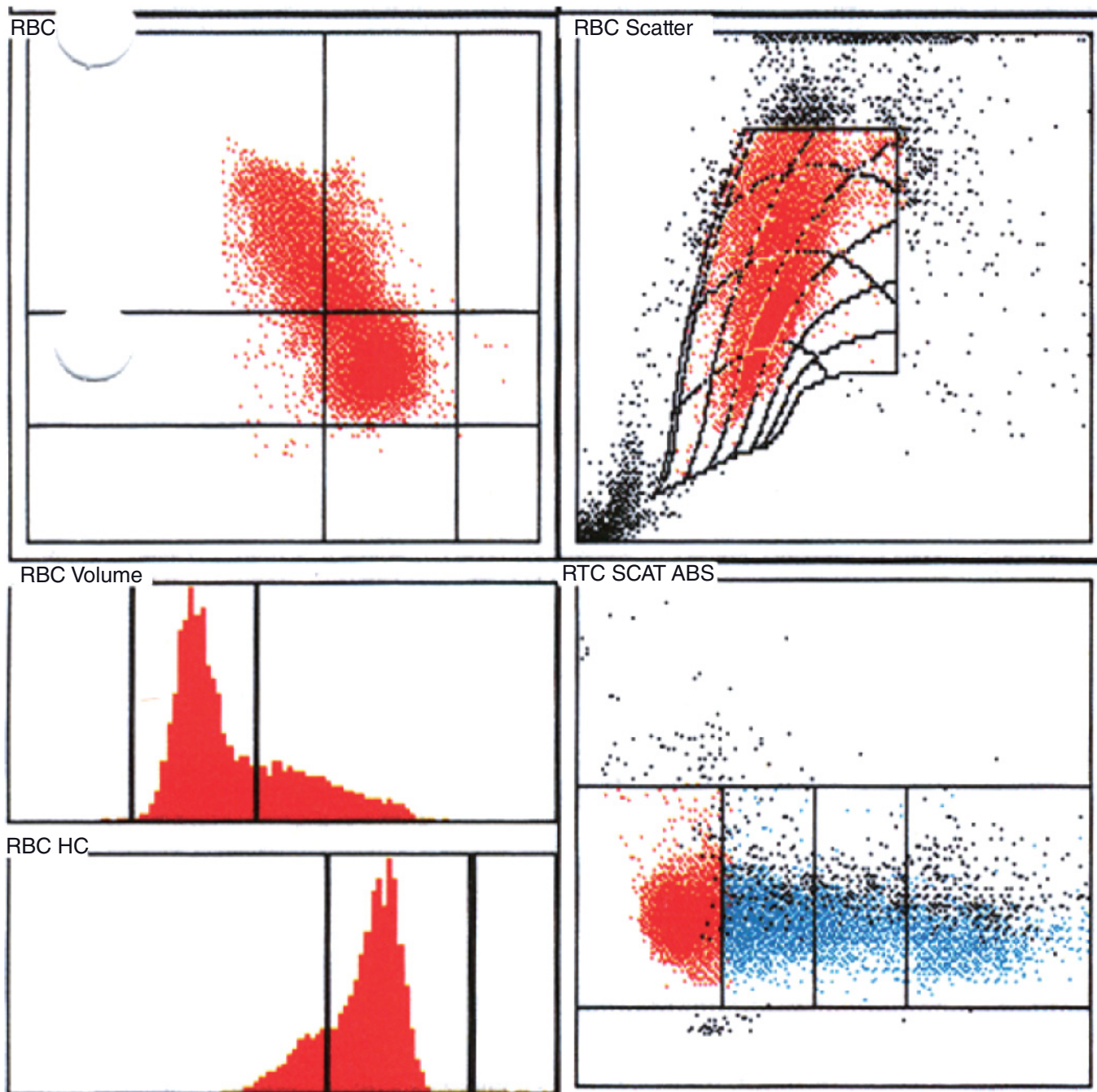


FIGURE 136.3 Advia 2120 graphics of a dog with a regenerative anemia and 21% reticulocytes. The lower right box shows reticulocytes as blue dots extending to the right of mature RBCs (red). The reticulocytes are divided into three groups based on RNA content. Those to the far right have the most RNA. The other four graphics show RBCs analyzed by size and hemoglobin concentration. These illustrate distinct populations of mature normocytic normochromic RBCs and immature macrocytic hypochromic RBCs.

HEINZ BODY STAINING AND COUNTING

Heinz bodies are formed from denatured, precipitated hemoglobin resulting from oxidative damage. Oxidation of sulfhydryl groups cause permanent damage and sulfhemoglobin formation (see Chapter 36).

Manual Methods

Heinz bodies may be seen on blood smears routinely stained with Romanowsky stains if they are large enough to bulge from the surface of the RBC or if they appear lighter and somewhat refractile compared to the rest of the hemoglobin in the RBC. Heinz bodies are

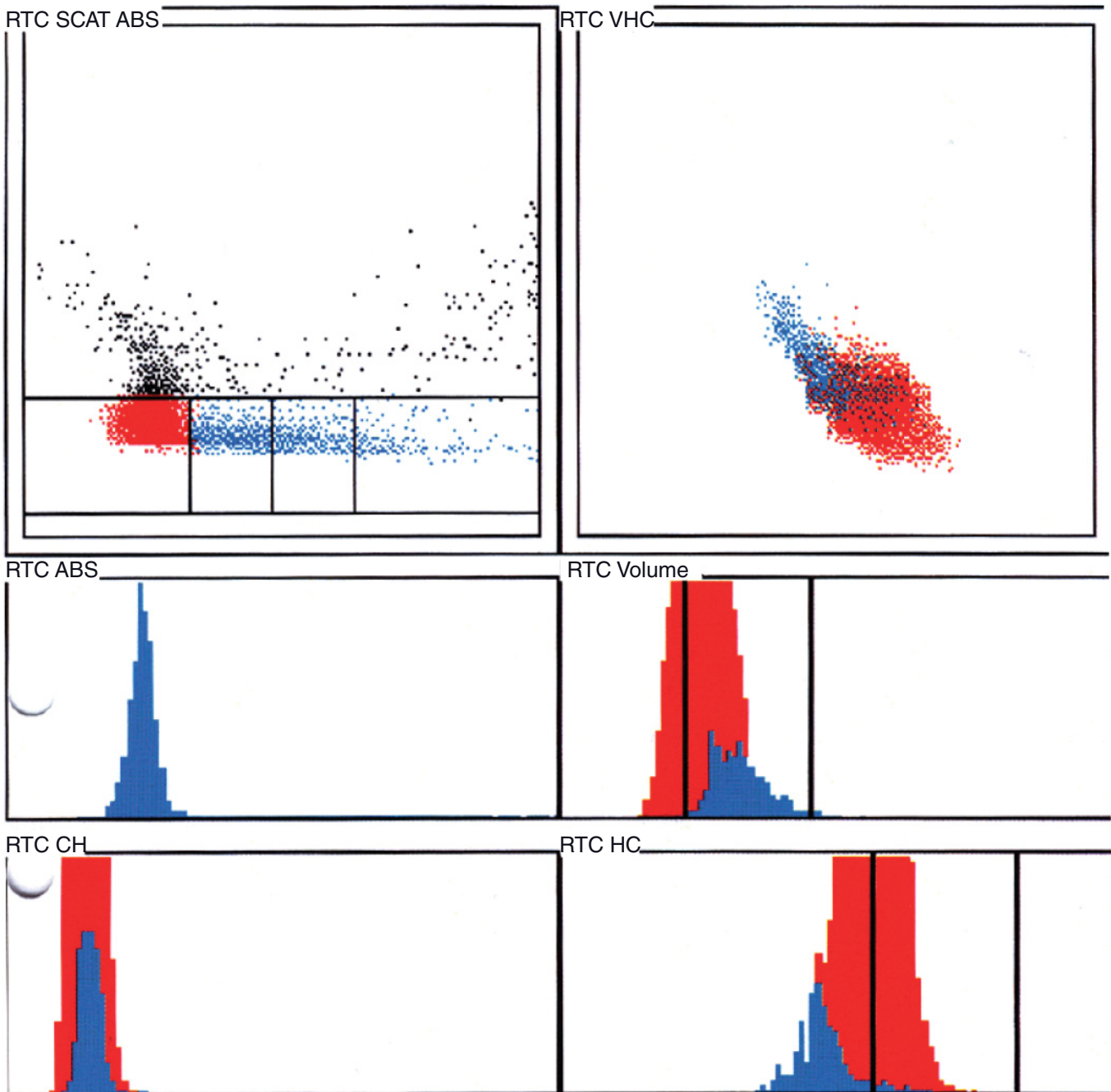


FIGURE 136.4 Advia 2120® reticulocyte graphics from a dog with iron deficiency. Note the reticulocyte (RTC) cluster in RTC SCAT ABS cytogram (upper left) is lower than the cluster in Fig. 136.3 reflecting that reticulocytes in iron deficiency anemia are smaller than normal reticulocytes. The RTC VHC cytogram, RTC volume histogram, and RTC HC histogram on the right show the blue reticulocytes were macrocytic and hypochromic when compared to mature RBCs.

clearly seen in ghost cells as hemoglobin colored bodies attached to the RBC membrane. Heinz bodies are best evaluated on blood smears stained for enumeration of reticulocytes (e.g. NMB- or BCB-stained smears). For birds, mix 2 drops NMB with 1 drop fresh EDTA blood and allow to stand at room temperature for 30 minutes before making the smear.⁶

Heinz bodies stain a lighter blue than ribosomes in reticulocytes. Heinz bodies may be small and numerous, and may be mistaken for reticulocytes. This erroneously affects the reticulocyte count and fails to detect the presence of Heinz bodies. Howell-Jolly bodies are nuclear remnants, stain a darker purple, and have a more distinct border than Heinz bodies. Howell-Jolly

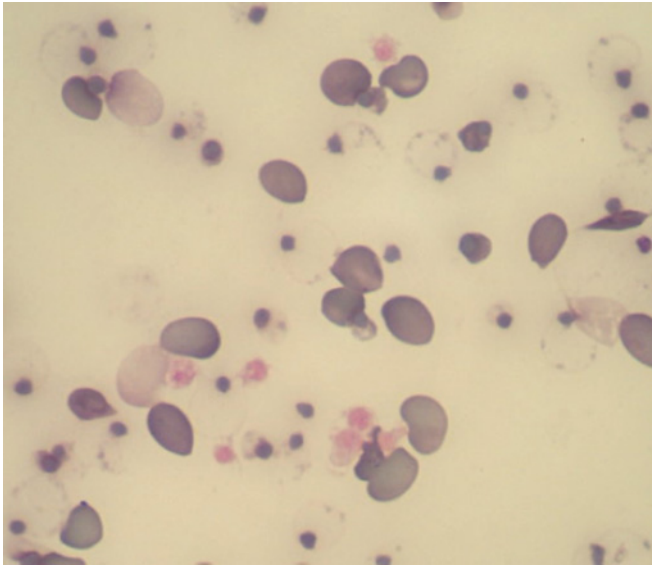


FIGURE 136.5 Modified Wright's-stained blood smear of a cat with onion toxicity. Many round Heinz bodies about the same color as hemoglobin are in ghost cells (lysed RBCs). One large Heinz body is usually seen in cats but small granular Heinz bodies are seen attached to membranes of some lysed RBCs.

bodies are usually too few in number to cause much error in manual Heinz body enumeration.

The total mass of precipitated hemoglobin (Heinz body material) is reflective of the magnitude of toxic injury; therefore the report should also indicate whether Heinz bodies were small or large, how much granular Heinz body material was also in the RBC and how much other damage to RBC was seen. Blood turbidity may be measured to estimate the amount of Heinz body material.⁷

Oxidative toxins that form Heinz bodies also cause other changes such as methemoglobin formation, eccentrocytes, ghost cells, and pyknotocytes. These other disease changes (and the presence of Heinz body induced artifacts, see below) should be checked for before a final hematology report is released from the laboratory. The presence and amount of those changes should be included in the report. The pattern and amount of change in Heinz bodies, eccentrocytes, and methemoglobin varies with different oxidative toxins and species.⁸

Heinz bodies in the blood sample may cause artifacts such as false increases in MCH and MCHC when determined by in laser hematology instruments, increased hemoglobin concentration when determined by spectrophotometric methods, and increased leukocytes in the basophil channel of the Advia® or earlier H-1® instruments (see Fig. 24.3, p. 156).¹⁰ The increased MCH and MCHC is due to increased optical density of RBCs containing Heinz bodies in the laser system of the Advia®. Heinz bodies interfere with the spectrophotometric measurement of hemoglobin by interfering with light transmission through the sample. This results in

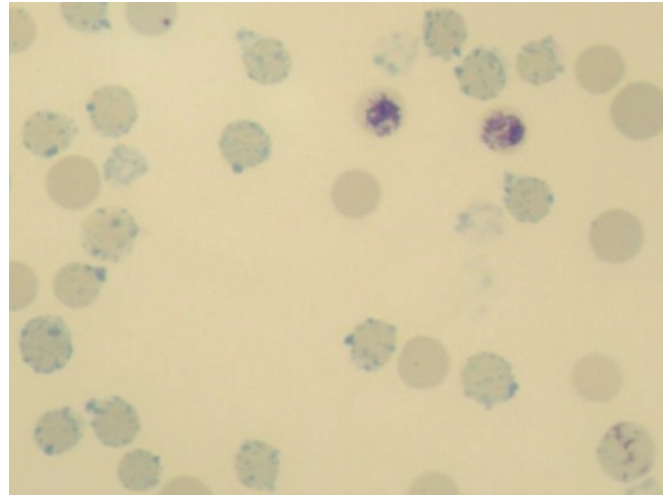


FIGURE 136.6 Brilliant cresyl blue-stained blood smear of a dog with onion toxicity. There are only two reticulocytes with dark blue-purple granules in the upper right. Most of the RBCs have many Heinz bodies which are lighter blue-stained granular bodies.

an overestimation of hemoglobin concentration. Heinz bodies in ghost RBCs in a cat gave a very erroneous basophil count of 69,750/ μL (Fig. 136.5).

Species Variations

The size of Heinz bodies varies greatly. Heinz described them as 1–2 μm in diameter in dogs, rabbits and guinea pigs, but largest in cats, in which they may be up to one-third the diameter of the RBC.¹¹ Heinz bodies in cats are predominately distinct, large round structures (Fig. 136.5). However, in dogs and other species they may be multiple, small granular bodies that can be mistaken for reticulocytes (Fig. 136.6).

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Flow Cytometry

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Instrumentation

- Essential Concepts of Flow Cytometers
- Basic Principles of Light Scatter and Fluorescence
- Principles of Cell Sorting

Sample Preparation

Sample Processing

Choice of Antibody and Fluorochrome

Sample Analysis

Instrument Maintenance

Applications in Veterinary Clinical Laboratories

- Erythrocyte Applications

- Erythrocyte indices

- Reticulocyte count and reticulocyte maturation index

- Erythrocyte-bound immunoglobulin

Platelet Applications

Leukocyte Applications

- Immunophenotyping of leukocytes

- Determination of clonality and DNA ploidy

- Evaluation of neutrophil function

Bone Marrow Applications

Cytopathology Applications

Conclusion

Acronyms and Abbreviations

FACS, fluorescent activated cell sorter; RBC, red blood cell; WBC, white blood cell.

Flow cytometry has emerged as a major technology that is now an essential component of veterinary clinical laboratories.⁹ Flow cytometric technology has been incorporated into hematology analyzers (see Chapter 135). This technology has facilitated more precise evaluation of red blood cell (RBC) size and hemoglobin content, determination of an automated white blood cell (WBC) differential cell count, and determination of several platelet parameters including detection of activated platelets. Flow cytometers, not associated with hematology analyzers, are also becoming an integral part of veterinary clinical laboratories, particularly in the area of preclinical safety assessment. These instruments are flexible and can be adapted to many cell and particle systems in which a single cell suspension can be produced and in which appropriate monoclonal antibody or specific cytofluorescent stain is available. Creating a single cell suspension is a relatively simple matter for most hemolymphatic tissues, including blood, bone marrow, lymph node, and spleen. Somewhat more complex processing, including mincing and protease digestion, is required to create single cell suspensions from solid tissues.^{2,6} The availability of species-specific monoclonal antibodies for domestic animals has limited the application of flow cytometry in veterinary medicine. However, the number of mono-

clonal antibodies available for cattle, sheep, pigs, horse, cats, and dogs from both commercial and private sources has expanded dramatically in recent years and many cross-reacting antibodies are also available.

INSTRUMENTATION

Flow cytometry is defined as a technology that measures multiple characteristics of cells or particles as they pass through a light source in a fluid stream.^{1,2,11} In addition to cells, flow cytometers can detect chromosomes, proteins, or molecules if they are attached to a particle such as a microsphere. A fluorescent activated cell sorter (FACS) is a flow cytometer that has the capacity to separate fluorescently-labeled cells from a mixed cell population. Most bench top flow cytometers do not have the capability to sort and are dedicated to analytical methods that measure light scatter and emitted fluorescent light.^{2,11}

Essential Components of Flow Cytometers

Analytical flow cytometers and cell sorters use a combination of fluidics, optics, electronics, and computer components in their analysis (Fig. 137.1). In the fluidics

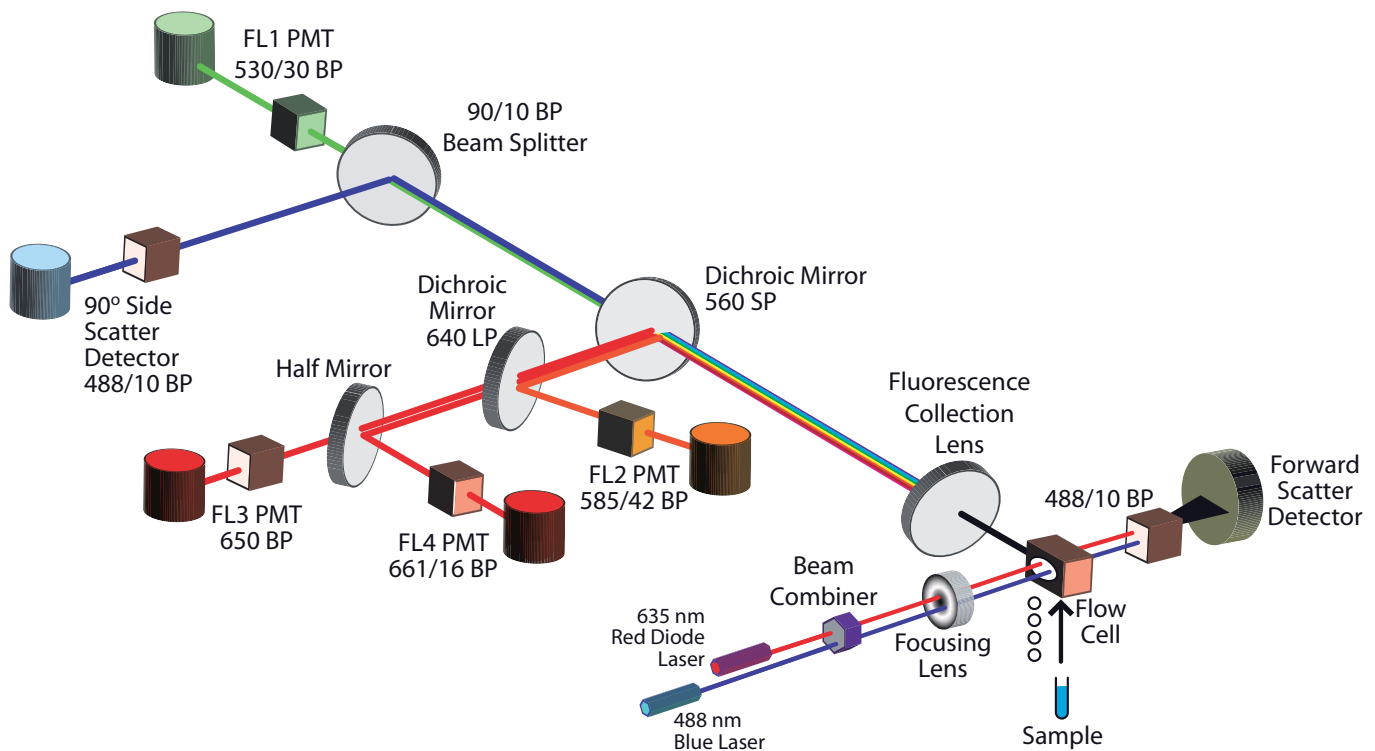


FIGURE 137.1 Schematic of the basic optical and detection components of a flow cytometer. PMT, photomultiplier tube; LP, light path.

system, cells suspended in a physiologic solution are mixed with a sheath fluid that is supplied in a pressurized reservoir. For most mammalian cells, the sheath fluid consists of a phosphate-buffered saline solution. The cell sample stream is at a higher pressure than the sheath fluid. As a result, the sample stream becomes a core in the sheath fluid, which is referred to as coaxial flow. This type of flow permits the cells in the sample stream to intersect the laser one cell at a time. This process is called hydrodynamic focusing. The pressure applied to the sample stream can be adjusted to alter the alignment between the cells and the laser beam. Increasing the sample pressure increases the flow rate by increasing the width of the sample core stream.

The optics component of the flow cytometer consists of a laser and a series of lenses to focus the beam. The number of lasers in a flow cytometer is critical to the number and type of fluorochromes that can be detected by the instrument. Argon gas is commonly used as the excitation laser. The energized light produced by these lasers emits at 488 nm. Many fluorochromes absorb at 488 nm light and emit light in the green (535 nm), orange (585 nm), and red spectrum (630 nm). However, ultraviolet (UV) sensitive or far-red fluorochromes require excitation lasers that emit light in the UV (300–400 nm) or far-red (630 nm) range. Other fluorochromes emit at a different wavelength and thus require separate detectors. The number of lasers, optical filters, and detectors determines the overall capacity of a given flow cytometer. Typical bench top systems, such as Becton Dickinson's FACSCalibur (Becton Dickinson, San Jose, CA), have two lasers (argon and red diode) and four

fluorescent detectors, thereby providing the possibility of four-color analysis. A typical optical bench from a two laser, four-color flow cytometer is depicted in Fig. 137.1.

Becton Dickinson's newer design in the FACSAria consists of an octagon arrangement of fluorescent detectors (photomultiplier tubes) that allow up to four lasers and 14 multicolor parameters.

Light signals detected by the detectors are converted to electronic voltage pulses. Forward-angle light scatter is converted to voltage pulses by a photodiode. Side-angle scatter and fluorescent light signals are converted to voltage pulses by photomultiplier tubes. Finally, voltage pulses are converted to a digital output and transferred to a computer.

Basic Principles of Light Scatter and Fluorescence

As cells pass single file through the laser beam, flow cytometers are capable of measuring two types of light scatter as well as detecting fluorescence.^{2,11} Forward-angle scatter light is laser light diffracted around the cell and is proportional to the size or volume of the cell. Light scatter in a 90° angle (side-angle scatter) is proportional to the internal complexity (i.e. granularity) of the cell. Based on these light scatter features, cells can be graphically displayed and separated based on their size and granularity. This is the principle used by laser-based clinical hematology analyzers to perform WBC differential cell counts (Fig. 137.2).

In addition to forward-angle and side-angle light scatter, flow cytometers are able to detect fluorescent

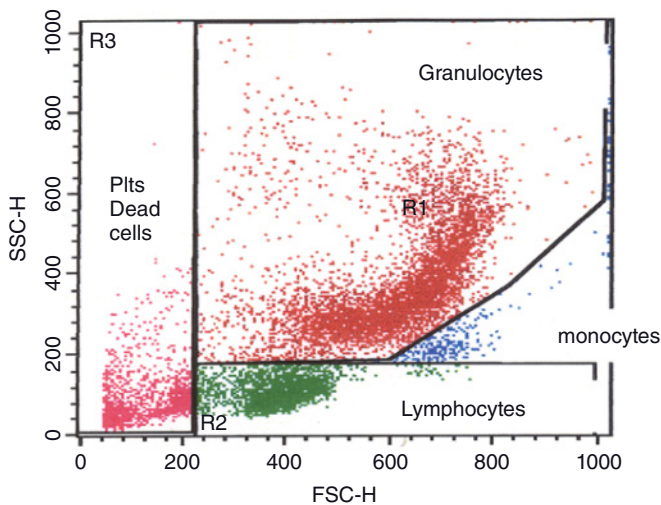


FIGURE 137.2 Forward-angle (FSC-H) versus side-angle (SSC-H) light scatter dot plots of blood leukocytes with gating used to perform differential WBC counts.

labels on cells. Depending on the capabilities of the flow cytometer, several fluorochromes can be detected simultaneously. This approach provides for identification of lineage-specific markers on a subset of cells in complex mixtures of cells or for detecting altered physiologic (i.e. activation) or pathologic (i.e. neoplastic, apoptotic) states of cells.

Principles of Cell Sorting

Cell sorters are flow cytometers that have the capacity to isolate a subpopulation of cells. Cell sorters have a nozzle tip that oscillates the sample core stream resulting in a stream that breaks up into droplets. Each droplet contains a cell. Typically the cell to be sorted is fluorescently labeled. As the droplets pass through the laser, the fluorescent label is detected and the droplet will be charged either positively or negatively by a charging electrode. Charged droplets travel toward positively or negatively charged high voltage platinum plates into a collection system (test tube, 96-well microtiter plate, glass slides etc.).^{2,11} The major advancement in recent years is ease of operation. The operation of current bench top analytical flow cytometers is relatively simple and permits opening access to multiple users once they have had some training. State of the art cell sorters still require a dedicated trained operator to operate and maintain the equipment.

SAMPLE PREPARATION

Collection and processing protocols vary with the cell type and assay method used.^{2,18} When analyzing blood cells, it is possible to analyze a whole cell sample and then focus (i.e. "gate on") on the cell population of interest. Alternatively, the cell population of interest can be isolated before analysis. Both approaches have advantages and disadvantages. Isolating a cell population can

be time-consuming and can introduce artifacts (e.g. cell activation, aggregation, altered cell size or shape, or even cell death). Alternatively, identifying a subpopulation of cells may require additional steps such as addition of one or two fluorescently-labeled monoclonal antibodies. Therefore, the blood cell collection and processing technique should be carefully researched to determine the most suitable method for a particular application.

White blood cells or WBC subpopulations can be isolated using density gradient centrifugation, or whole blood specimens can be used. Presently, the whole blood method is used most frequently because it is faster and easier to perform and avoids cellular alterations associated with density gradient centrifugation. Red blood cells can be removed by addition of an RBC lysis buffer. Commercially available lysis buffers come with a cell fixative added. Fixation prevents activation and clumping of WBCs and does not adversely affect binding of most antibodies. If tests of neutrophil function are performed, whole blood may be preferred to prevent in vitro neutrophil activation during processing (see Chapter 141). Red blood cells can be analyzed using anticoagulated whole blood or RBCs collected from the RBC layer of cells after centrifugation of whole blood. Bone marrow and solid tissue require somewhat more elaborate processing procedures (see Chapter 132). Platelet-rich plasma is most frequently used for platelet analysis. Platelet-rich plasma is prepared by low speed centrifugation of anticoagulated whole blood. Platelets become activated and clump when pelleted. Therefore, platelet function must be inhibited for most flow cytometric applications. Various methods of platelet function inhibition, including paraformaldehyde fixation, addition of prostaglandin E1, and lowering pH to 6.5, have been used.

SAMPLE PROCESSING

A large spectrum of flow cytometric tests have been described for analysis of blood cells. A basic method of preparation is common to most assays. In general, these techniques involve incubation of cells, typically for 30 minutes, with a monoclonal antibody or fluorochrome stain directed against a cellular antigen of interest. If this antigen is inside the cell, cells may need to be permeabilized to permit the marker to enter the cell. If the monoclonal antibody is labeled with a fluorescent dye, the cell suspension is washed, resuspended in buffer solution, and analyzed. If the primary antibody is not labeled, a labeled secondary antibody, typically an anti-mouse immunoglobulin antibody is added. The secondary antibody binds to the first antibody in proportion to the amount of primary antibody that is bound. After removing excess unbound secondary antibody by washing, the specimen is analyzed. The number of cell washes used varies between investigators.

Samples should always be inspected grossly or microscopically for cell clumps. Cell clumps can readily plug flow cytometers. Clumping of the sample can be

greatly reduced by adhering to optimal cell concentrations of no greater than 1×10^6 cells/mL. Cell clumps can be removed from samples by filtering the sample through special filters with $30\mu\text{m}$ pores that are designed to fit into tops of 6 mL tubes.

CHOICE OF ANTIBODY AND FLUOROCROME

The spectrum of species-specific and cross-reacting antibodies available for species of interest to veterinary medicine has increased dramatically in recent years. The choice of antibody for cell labeling is of critical importance to successful flow cytometric procedures. Some antibodies work well for flow cytometric procedures while others do not. Many commercially available antibodies have been tested for use in flow cytometric applications. A partial list of several references that have used species-specific and cross-reactive antibodies is available in Chapter 4. If the antibody has not been tested in the species of interest, the commercial antibody should be tested at serial dilutions to determine the optimal titer at which the nonspecific binding is minimum, specific antibody binding is maximum, and therefore the fluorescence signal-to-noise ratio is at its highest.³ Binding of some antibodies to cells may be affected by processing conditions such as cell fixation, while other antibodies may not be affected. Many antibodies are nonspecifically taken up by dead and dying cells resulting in false positive labeling.

The choice of fluorochrome is also important. The choice is limited to which fluorochrome the excitation lasers can excite. Users should always verify before purchase that the fluorochrome of interest can be excited by the exciting lasers. Fluorescein is a commonly used label that has the advantage of being highly stable; however, it has high background fluorescence. The red emitters (e.g. PerCy5, PerCP), are somewhat less stable but have a lower background fluorescence.

SAMPLE ANALYSIS

A few general comments about operation of flow cytometers will be presented. Cells are typically initially displayed as forward-angle versus side-angle light scatter dot plots. Most frequently, side scatter (i.e. granularity) is on the y -axis and forward scatter (i.e. size) is on the x -axis. Voltages are adjusted by the operator until the cells of interest are centered in the dot plot. Although it is logical to think that each dot on the dot plot identifies one cell, each dot actually identifies all cells with the same scatter properties. Therefore, one dot could represent one cell or many cells. Gates can be set around cell population of interest to quantify them.

If cells are fluorescently labeled, fluorescence can be detected in a separate plot. Fluorescence intensity (on a log scale) is usually plotted against forward-angle light scatter. Histogram plots or dot plots can be used. The

most critical determination to be made is that of which cells are fluorescing. This is complicated by the fact that all cells have some level of background fluorescence and the apparent fluorescent intensity of a cell population can be changed by adjusting the gain setting of the instrument. Some fluorescent labels have a relatively high level of background fluorescence that is greater if samples have been exposed to light. Background fluorescence is relatively high for fluorochromes that are green, yellow, and orange emitters. Alternatively, red emitters have comparatively low background fluorescence. To help the operator set a gate between fluorescent and non-fluorescent cell populations, several controls should be used. These include a negative control incubated with an irrelevant isotype-matched control antibody and positive-labeled control. The negative control specimen frequently cannot be adjusted to zero because of nonspecific antibody binding and autofluorescence, so values are typically adjusted to a value of <10 channel units. If a positive control is not available, fluorochrome-labeled beads can be substituted in some applications.

If two or more fluorescent markers are used in a single sample, the emission spectra of the fluorochromes may overlap. For example, if cells are labeled with fluorescein (FL1 emission peak at 530 nm) and phycoerythrin (FL2 emission peak at 575 nm), the detector for fluorescein will detect some of the phycoerythrin in the sample and vice versa. To compensate for this overlap of fluorescence, the operator puts a sample in labeled only with fluorescein (green fluorescence) and adjusts the FL2 detector to subtract out the percentage of fluorescein that is detected (i.e. typically to <10 channel units). A sample labeled only with phycoerythrin is then added and the FL1 detector is adjusted remove the phycoerythrin that is detected.

After this quality control procedure is performed, a dual-labeled or multi-color sample can be acquired and analyzed with confidence and accuracy. There is software available that will allow the user to compensate for fluorescence overlap after acquisition (WinList™ Verity Software).

Threshold setting permits the operator to set a limit below which data will not be detected. Setting a higher forward-angle and side-angle light scatter threshold eliminates small particulate debris and/or platelets from the analysis. If the threshold is raised to 200, platelets will be excluded from acquisition or analysis of the preparation. Threshold setting for fluorescence intensity eliminates nonspecific fluorescence. Threshold settings frequently improve the results obtained.

INSTRUMENT MAINTENANCE

Technical advances in design and computerization of flow cytometric instrumentation has opened the use of these instruments to multiple users including laboratory staff, researchers, and graduate students. Flow cytometers are highly adaptable to many different techniques and the operator has great flexibility in manipu-

lating the appearance of the computer-generated graphics and the final results obtained. As such, the operator has the capacity to produce valid or nonsensical results.

All results obtained are dependent on the quality of routine maintenance, calibration, and troubleshooting. While it is possible to have multiple people operating a particular flow cytometer, it is essential to have one responsible person in charge of calibration, maintenance, and troubleshooting. Maintenance and calibration are instrument dependent and will not be discussed in detail here. In general, they involve daily calibration using fluorescent beads, a checklist of routine maintenance, a troubleshooting guide, a protocol for start up and shut down each time the instrument is used, and a maintenance contract with the manufacturer.

APPLICATIONS IN VETERINARY CLINICAL LABORATORIES

The potential applications of flow cytometry are diverse.¹⁵ In this chapter, we will list some techniques that provide clinically relevant data or provide more valid or more quantifiable data than are presently available. Some of these tests are discussed in more detail in other chapters in this book.

Erythrocyte Applications

Major applications of flow cytometry to RBCs include determination of RBC indices on individual cells, determination of reticulocyte count and reticulocyte maturation index, and determination of anti-RBC antibody binding.

Erythrocyte Indices

Newer flow-based hematology analyzers determine the size and complexity of individual RBCs and provide RBC histograms. This provides much more clinically relevant information than previous measures of RBC indices (see Chapter 135).

Reticulocyte Count and Reticulocyte Maturation Index

The manual method of reticulocyte counting is relatively imprecise, with coefficients of variation of 8% to 23% reported for dog blood.¹² Flow cytometry is a more precise method for reticulocyte counting. The technique is discussed in Chapter 136.

Erythrocyte-bound Immunoglobulin

The direct anti-globulin (i.e. Coombs') test has been routinely performed in veterinary laboratories. However, the sensitivity is relatively low being in the range 48–58% when performed by the standard tube

method (see Chapter 140).^{10,23} More recently, flow cytometric techniques have been developed for detection of erythrocyte-bound immunoglobulin in dogs and horses.^{10,23} In two studies, this resulted in sensitivity of 92% for diagnosis of immune-mediated hemolytic anemia. In this technique, washed RBCs were incubated with fluorescein isothiocyanate (FITC)-labeled sheep-antidog IgG, FITC-labeled goat-antidog IgM, or FITC-labeled goat-antidog C3. Cells were washed, resuspended and fluorescence analyzed in a flow cytometer. These results indicate that the flow cytometric test is more sensitive than the Coombs' test, but may also be somewhat less specific.

Platelet Applications

Clinical applications of flow cytometry for evaluation of platelets include quantitation of immature (i.e. reticulated) platelets, detection of anti-platelet antibody, and detection of activated platelets. Immature platelets have been quantified in blood from humans, dogs, and horses in much the same way that reticulocytes are analyzed.^{13,22} Immature platelets contain greater RNA content compared to mature platelets and this residual RNA can be quantified by incubating platelets with the fluorescent stain thiazole orange. The percentage of platelets with increased fluorescence intensity can then be determined (see Chapter 135).²²

Flow cytometry has been used to detect anti-platelet antibody in a manner similar to that for detection of anti-RBC antibodies.⁷ This test method is discussed in Chapter 140.

A variety of flow cytometric tests have been developed to detect circulating activated platelets. These include binding of fibrinogen to platelets, binding of monoclonal antibodies to activation-dependent markers on the platelet surface, detection of platelet microparticles, detection of platelet shape change, and detection of platelet-leukocyte aggregates (see Chapter 142).^{1,4,21} Activation markers detect conformational changes in surface proteins (e.g. activated glycoprotein IIb/IIIa), translocation of granule-associated proteins to the platelet surface (e.g. P-selectin, glycoprotein 53, dense granule membrane protein), or binding of secreted proteins to the platelet surface (e.g. thrombospondin). The activation-associated conformational change in glycoprotein IIb/IIIa can be detected by quantifying fibrinogen bound to the platelet surface or by addition of antibodies that specifically react with activation-associated binding sites. Few antibodies are available for use in animals. Anti-human fibrinogen cross-reacts with cat, horse, and pig, but not with dog.^{4,21} A monoclonal antibody that recognizes a receptor-induced binding site on canine fibrinogen has been described.¹ A monoclonal antibody that reacts with canine P-selectin also has been described.⁴

Platelet microparticles can be measured as an indication of platelet activation. Platelet microparticles are vesicles derived from the plasma membrane of activated platelets. Platelet microparticles can be detected by centrifuging citrate-anticoagulated blood at

$10,000 \times g$.²⁴ Centrifugation results in nearly complete removal of platelets. The platelet-poor plasma is then incubated with a platelet membrane-specific antibody, such as anti-glycoprotein IIb/IIIa. Thereafter, the number of fluorescent particles is quantified. Increased numbers of platelet microparticles have been reported in pigs during experimental endotoxemia.⁵

Activated platelets adhere to leukocytes.²¹ Adhesion is through expression of P-selectin (i.e. CD 62) on the surface of activated platelets and its interaction with platelet-specific glycoprotein ligand on the surface of leukocytes. P-selectin is normally expressed on the internal surface of alpha granules but, upon degranulation, is transported to the platelet surface. Activated platelets primarily bind to neutrophils and monocytes. The test is usually done using heparinized whole blood, because platelet-leukocyte adhesion is calcium dependent.²¹ Prostaglandin E1 can be added to prevent post-collection platelet activation.²¹ The assay is based on fluorescent labeling of platelets in whole blood or more frequently in whole blood after erythrocyte lysis. Cells are then displayed as forward-angle versus side-angle scatter plots, and the neutrophils and/or monocyte populations are gated and analyzed for fluorescence intensity. Fluorescence of leukocytes is an indication of binding of platelets to the leukocyte surface. Increased numbers of platelet-neutrophil aggregates have been identified in horses during intensive treadmill exercise²⁰ and in ponies during the prodromal stages of alimentary laminitis.²¹

Leukocyte Applications

A variety of flow cytometric tests have been described for evaluation of leukocytes. Of these, analysis of lymphocyte subtypes and immunophenotyping of hemic neoplasia have found clinical applications.⁶ Neutrophil function tests have been described and have been used extensively for research studies but it is unclear if these tests will find clinical applications other than detection of the relatively rare hereditary neutrophil function defects (see Chapter 141).

Immunophenotyping of Leukocytes

Immunophenotyping of leukocytes involves the use of antibodies and fluorochrome reagents to identify specific cell types in blood, bone marrow, or solid tissues. Fluorochromes are generally not species-specific and, therefore, can be used for a variety of species. Alternatively, antibodies that detect specific markers on cell membranes tend to be species-specific. This technique is particularly useful in performing differential cell counts on bone marrow (see Chapter 132), in classifying myeloproliferative and lymphoproliferative diseases (see Chapter 143), and in evaluating immune dysfunctions associated with lentivirus and feline immunodeficiency virus infections (see Chapter 55) and associated with rickettsial and autoimmune diseases (see Chapters 33 and 34).

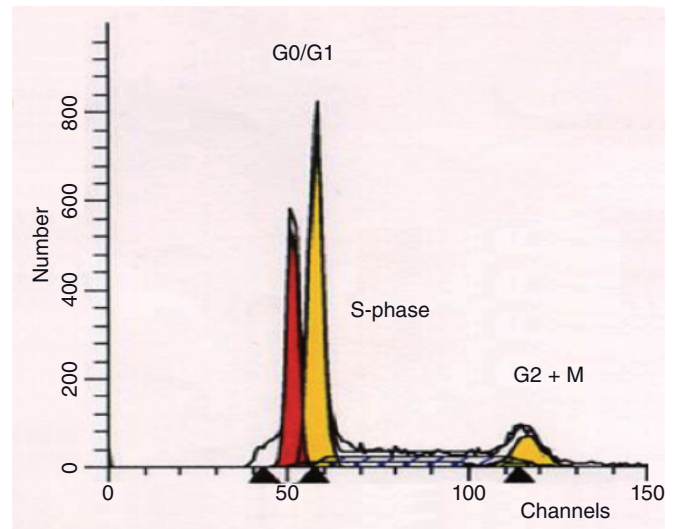


FIGURE 137.3 Analysis of DNA ploidy and cell cycle determination by use of flow cytometry. Peritoneal fluid from a horse was analyzed. Yellow peaks represent aneuploid DNA that is an indication of tumor-associated alterations in chromosome number.

Determination of Clonality and DNA Ploidy

In addition to evaluation of the immunophenotype of potentially neoplastic populations, markers of clonality and DNA ploidy can be determined as indicators of malignancy (see Chapter 143). Clonality is an indication that cells are progeny of a single cell and therefore likely represent a malignant population. For B-cell malignancies, the exclusive presence of kappa or lambda immunoglobulin light chain rather than a mixture of the two indicates clonality. Likewise, the presence of a specific T cell receptor rearrangement indicates clonality of T cell malignancies. DNA ploidy and cell cycle analysis can also be used to identify malignant cells (Fig. 137.3). Peaks in DNA histograms, in addition to the normal diploid peak, are an indication of tumor-associated alterations in chromosome number. Cellular DNA is usually stained with the fluorochrome propidium iodide.¹¹

Evaluation of Neutrophil Function

A variety of flow cytometric tests have been described to define neutrophil function (see Chapter 141). These tests include oxidative metabolism,² phagocytosis, degranulation, activation markers, surface expression of secreted granule contents, shape change, F-actin content, and opsonization.

Bone Marrow Applications

Flow cytometric techniques have been used to determine bone marrow total and differential cell counts, determine megakaryocyte maturation, quantify the rate of cell proliferation, and detect and differentiate leukemias and myelodysplastic syndromes. Several

approaches have been used to perform bone marrow differential cell counts. Rat bone marrow differential cell counts were done after labeling cells with the fluorochrome 2',7'-dichlorofluorescein-diacetate.³ This reagent preferentially labels peroxidase-positive cells. In the presence of hydrogen peroxide, peroxidase converts 2',7'-dichlorofluorescein-diacetate to the fluorescent dichlorofluorescein compound. The technique is relatively simple. Anticoagulated marrow samples are incubated with 2',7'-dichlorofluorescein-diacetate for 15 minutes, hydrogen peroxide is added and incubation is continued for an additional 15 minutes. Marrow specimens are analyzed as log green fluorescence versus forward-angle light scatter plots. When compared to manual differential cell counts, this technique was able to identify populations of proliferating and mature myeloid cells, proliferating and mature erythroid cells, and megakaryocytes.³ The technique was not able to differentiate lymphoid cells from mature erythroid cells. Therefore, the technique was combined with the use of monoclonal antibodies to detect B and T cells. Bone marrow of several species has also been analyzed by use of the lipophilic cationic dye 3,3'-dihexyloxacarbocyanine iodide.⁸ The technique was simple, involving incubation of bone marrow aspirate material with the dye for 30 minutes and analysis as log green fluorescence versus forward-angle light scatter plots. Populations of mature and immature myeloid cells, mature and immature erythroid cells, and lymphocytes were detected. The technique gave acceptable differential cell counts for mouse, rat, dog, and cynomolgus monkey.⁸

Canine and feline bone marrow was evaluated by use of forward-angle versus side-angle light scatter plots.^{16,19} This relatively simple approach gave acceptable identification of mature and immature granulocytes and mature and mature erythroid cells, but failed to identify lymphocytes or monocyte/macrophages. Identification of subpopulation within the bone marrow was further refined through use of lineage-specific monoclonal antibodies.¹⁷ Canine bone marrow was also evaluated by labeling bone marrow with anti-dog CD45 and analyzing cells in scatter plots as side-angle light scatter versus fluorescence intensity.¹⁸ Populations of mature granulocytes, myeloblasts, erythroid precursors, lymphocytes, and monocyte/macrophages were identified.

A method for bone marrow total and differential cell counts in preclinical safety assessment studies for rats, mice, monkeys and dogs is described in Chapter 132. It involves using a combination of anti-CD45 and anti-transferrin receptor (CD71) antibodies. This is coupled with side scatter for cellular complexity and nucleic acid staining with LDS-751 for separation of nucleated cells from RBCs.

Megakaryocyte ploidy can be analyzed by use of flow cytometry. Megakaryocytes are labeled with a megakaryocyte-specific monoclonal antibody and DNA is labeled with propidium iodide. Monoclonal antibody-associated fluorescence is used to identify megakaryocytes and the intensity of propidium iodide

staining varies directly with the number of nuclei present in the cells.

Proliferative activity of bone marrow has been measured by use of the fluorescent DNA stain propidium iodide or by staining with bromodeoxyuridine.¹⁴ Mitotic activity causes slight variations in the DNA content of cells that result in altered fluorescence. Bromodeoxyuridine is incorporated into mitotic cells. An anti-bromodeoxyuridine antibody is needed for flow cytometric analysis. Both the frequency of mitosis and the stage of mitosis can be determined. The proliferative rate of a specific population of bone marrow cells can be determined by labeling the population with a specific monoclonal antibody and with propidium iodide.¹⁴

Cytopathology Applications

Flow cytometry has been used to analyze cell from tissues other than blood and bone marrow. Techniques for creating a single cell suspension vary with the tissue studied.⁶ Because of the extensive processing required for some tissues, staining and examination of cells before analysis is essential to establish that the cell type of interest is present and that the cells are intact. Flow cytometry has been applied to evaluation of cytologic tissue from many tissues including lymph node, urine, semen, breast, lung, liver, pancreas, and endocrine tissue.⁶ Flow cytometric analysis has been used almost exclusively for detection and differentiation of malignancies (Fig. 137.3). The only test frequently applied to cytologic specimens is DNA analysis for aneuploidy and proliferation rate. However, tests for clonality and cell lineage determination have been applied to lymph node aspirates.⁶

CONCLUSION

Flow cytometric technology has been incorporated into conventional hematology analyzers, and as such has become part of many veterinary clinical laboratories. Flow cytometers, not associated with hematology analyzers, are in routine use in preclinical safety assessment laboratories, research laboratories, and in some clinical laboratories. As instrumentation, reagents, and demand for flow-based testing continue to advance, use of these instruments should continue to increase.

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Laboratory Testing of Coagulation Disorders

GEORGE LUBAS, MARCO CALDIN, BO WIINBERG, and ANNEMARIE T. KRISTENSEN

- Methodology of Plasma Based Hemostasis Tests
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- Tests for the Monitoring of Anticoagulants
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 - Heparin Assays
- Functional Tests for Fibrinolysis
 - Plasminogen
 - Alpha-2-Antiplasmin
 - Plasminogen Activators
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- Tests for Hemostasis Activation
 - D-Dimer
 - Fibrin/Fibrinogen Degradation Products
 - Thrombin Anti-Thrombin Complex
 - Prothrombin Fragment F 1+2
 - Fibrinopeptide A
 - Soluble Fibrin
- Thromboelastography
 - Method
 - Clinical Application

Acronyms and Abbreviations

ACT, activated clotting time; Ag, antigen; APC, activated protein C; APCR, activated protein C resistance; α 2-AP, alpha-2-antiplasmin; aPTT (or PTT), activated partial thromboplastin time; AT, antithrombin; Ca^{2+} , calcium ions; DDAVP, 1,8-desamino-d-arginine vasopressin or desmopressin; DIC, disseminated intravascular coagulation; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme linked immunosorbent assay; F 1+2, prothrombin fragment F 1+2; FVII, coagulation factor VII, and so on; FXa coagulation factor X activated, and so on; FII, prothrombin, FI, fibrinogen; FDPs, fibrin/fibrinogen degradation products; FM, fibrin monomers; FPA, fibrinopeptide A; FPB, fibrinopeptide B; HMWK, high molecular weight kininogen; HS, heparin sulfate; INR, international normalized ratio; ISI, International Sensitivity Index; IV, intravenous; LMWH, low molecular weight heparin; NPT, near patient testing; PAI-1 or 2, plasminogen activator inhibitor 1 or 2; PC, protein C; PL, phospholipids; pNA, para-nitroaniline; PPP, platelet-poor plasma; PR, prothrombin ratio; PS, protein S; PT, prothrombin time; SF, soluble fibrin; SLE, systemic lupus erythematosus; TAT, thrombin anti-thrombin; TEG, thromboelastography; TF, tissue factor; TM, thrombomodulin; t-PA, tissue plasminogen activator; TT, thrombin time; UFH, unfractionated heparin; u-PA, urokinase plasminogen activator; VWD, von Willebrand disease; VWF, von Willebrand factor.

METHODOLOGY OF PLASMA BASED HEMOSTASIS TESTS

Blood Sampling

Proper blood sampling is critical to hemostasis investigation in all species. Ideally, drawing the blood into a plastic syringe containing the appropriate amount of citrate for the volume of blood should be used. As this procedure is not always easy to perform, blood is drawn and then transferred to the citrate tube. Blood sampling through non-heparinized catheters may be done, if necessary, after flushing the catheter with at least 5 mL of saline solution (or six times the catheter dead space) and then discarding the first 5 mL of blood collected.¹¹⁴

Blood sampling through heparinized catheters generally should be avoided, though a flush and discard procedure as above in healthy dogs produced similar results as samples collected from direct venipuncture.⁹³

As a general rule, care should be taken to minimize platelet or coagulation and fibrinolytic system activation. Blood should be collected by atraumatic venipuncture on the first attempt, allowing a free flow into the syringe and avoiding unnecessary vacuum as turbulence may activate platelets. If collection is difficult, as in the case of sampling through a hematoma or exiting and re-entering the vein, a new vein site should be selected. Discarding a few drops of blood or using two tubes (the former for other purposes, the latter for coagulation studies) could be standardized, but this is not necessary if a clean venipuncture is obtained.¹³³

In the plasma based assays, the sample is citrated plasma that is harvested either from siliconized glass or plastic tubes. In humans, statistically significant differences in test results have been documented in comparisons of plastic versus glass tubes, but these differences are small and rarely of clinical importance.⁷⁰ The blood collected should be anticoagulated with trisodium citrate at an anticoagulant to blood ratio of 1:9 (by using 3.2% or 3.8% citrate tubes according to the laboratory preferences). Minimal influence on assay results in healthy dogs or dogs with hereditary hemostatic disorders have been documented using these two citrated formulated tubes.^{108,135}

It is important to maintain the 1:9 citrate to blood volume ratio. Overcitrated samples (e.g. in case of erythrocytosis where there is less plasma with the same amount of citrate) may have reduced coagulation activity or prolonged times because of the excess of citrate. Undercitrated samples (e.g. in case of severe anemia where there is more plasma with the same amount of citrate) may be hypercoagulable or show reduced times. Even the hematocrit of the sample may influence the hemostatic assays, and the correction of the citrate amount has been suggested in humans according to the following formula: citrate volume in mL = $0.002 \times (100 - \text{hematocrit value}) \times \text{volume of collected blood}$.^{83,114,130,133}

It has been shown recently that EDTA samples can be considered as a possible suitable alternative to citrated samples for some hemostasis testing. Assays should be performed as soon as possible after the sample collection, and appropriate reference intervals are needed.^{23,42}

Sample Processing and Storage

After confirming the absence of any clots in the sample, the citrated blood should be centrifuged for 10–15 minutes at $1500 \times g$. The general recommendation is to centrifuge and remove the plasma within 1 hour and test it within 4 hours from the drawing. The supernatant platelet-poor plasma should be transferred to plastic tubes by using plastic pipettes.¹¹⁴ Recently, it has been documented that is possible to analyze human blood specimens for several coagulation assays in the original collection tube after 24–48 hours, without observing clinically relevant changes.¹⁶⁶

Storage of canine citrated plasma samples collected from healthy animals yielded stable hemostasis results for 48 hours when stored at 24°C (even better than samples kept at 4°C), except for a mild decrease of fibrinogen at 24–48 hours. This evidence is important for samples that should be shipped to a referral laboratory. Similar studies are lacking in other species.³⁹

In the case of frozen plasma samples it is advisable to test them promptly after thawing, and the process of thawing should be rapid to minimize cryoprecipitate formation.¹³³

Coagulometers

Coagulometers can be manual, semiautomatic, or automatic devices (Fig. 138.1) depending on the operational procedure available in the laboratory. Manual coagulation testing, such as the tilt tube or manual hook method, are seldom used.⁶⁷

Introduction of semi or fully automated coagulometers has improved precision, reduced operator variability, and has resulted in significant cost and time savings. These coagulometers employ various physical clot detection methods such as mechanical by a steel ball, turbidimetry, and nephelometry or light scatter, or a combination of the above methods.^{56,68,129}

In the coagulometer using mechanical end-point detection, the sample and reagents are dispensed into a cuvette, which holds a disposable little steel ball. The ball rotates and a magnetic sensor detects the change of the magnetic field, which occurs when the forming fibrin strands interfere with the rotation of the ball.^{56,67,104,151}

The coagulometer using optical end-point detection of fibrin is based either on turbidimetry or nephelometry (detection of light scatter). In addition to the clotting assays, the detectors can also be used for turbidimetric immunochemical assays, and, in the case of turbidimetry, the use of specific filters in combination with the light source of the instrument allows chromogenic assays to be performed. The time elapsed until the

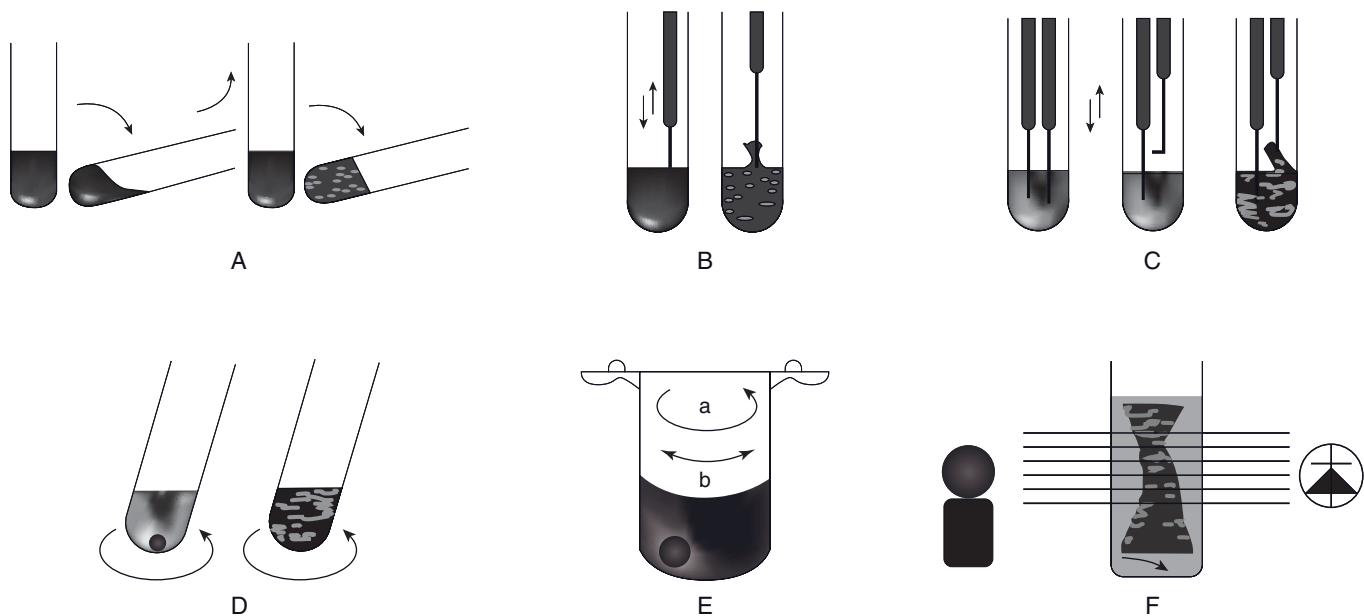


FIGURE 138.1 Clot Detection Methods. (A) The manual tilt tube method provides a visual clot endpoint.¹²¹ (B) The manual hook method uses a hook to lift the clot out of the sample, indicating the endpoint.⁶⁸ (C) The hook mechanical coagulometer.¹²⁹ It employs two electrodes. Before clot formation, the electrical circuit is made with both electrodes immersed in the plasma sample (left) and interrupted when the hook is lifted (center). When the clot is formed (right) the fibrin clot maintains the conductivity and the endpoint time is recorded. (D) The ball coagulometer, rotational technique. The steel ball is kept stationary both by gravity and a magnet at the bottom while the tube is rotating. During clot formation the electromagnet detects the change of the magnetic field, which occurs when the forming fibrin strands interfere with the movement of the ball on one side. (E) The ball coagulometer, oscillational technique. The steel ball is oscillated in the tube with the plasma sample. The ball movement is secured by a magnet. When the fibrin clot forms the ball stops moving and the endpoint time is recorded. (F) Clot detection in an optical and mechanical coagulometer. The source of light (tungsten lamp) at the left is continuously adjusted to give a constant voltage at the photodiodes located at the right. When the voltage change exceeds predetermined limits, the clotting time is recorded.

signal begins to change is the “clotting time.” Depending on the instruments, different algorithms are used for the calculation of the end-point and the threshold is set at different ways, e.g. end-point is set when a total change of absorbance/scatter of 50% has been reached or when the time for a predefined change of absorbance has occurred.^{56,67,104,151}

All clot detection methods have their limitations – optical devices suffer when lipemic or icteric samples are used, while mechanical devices are critically influenced by lipemia.^{56,67,104, 151}

Clotting Assays

Clotting or chronometrical assays, usually performed with a coagulometer, are still the most often used tests for hemostasis in veterinary medicine. Indeed, these tests are mostly used for preoperative screening, evaluating an animal suspected of having a coagulation disturbance, for monitoring therapy, and to screen function of entire pathways or of the individual components of the pro- and anticoagulant pathways.^{31,43,151}

A clotting assay measures the time in which the mixture of sample and reagents starts to clot, after addition of a starting reagent. For precision reasons, clotting assays should be developed so that the clotting times

are in the typical measuring ranges of more than 5–6 and less than 30–50 seconds. If the assays are to be adapted to optical analyzers, agents which improve the optical clot opaqueness may be used in order to enhance the signal.^{31,43,104,151}

Activators

Coagulation factors in plasma are usually inactive and require proteolytic activation. The activators of choice are: unphysiological substances such as silica, kaolin, and celite; physiological substances such as tissue extracts or purified or recombinant activated coagulation factors; or specific snake venom derived enzymes. Several of the enzymatic steps require not only the activated coagulation factors, but also an activated co-factor, phospholipids (PL) and Ca^{2+} . This means that a very complex mixture of relatively unstable proteins, which are sometimes difficult to purify and to activate themselves, needs to be stabilized. The use of highly specific snake venom enzymes, derived from the venom of many species, is often a better approach than the use of natural activators. These products are often resistant against the plasma protease inhibitors or direct anticoagulants of the patient plasma. Further, many are not dependent on co-factors, not even on Ca^{2+} .^{31,43,104,151}

Chromogenic and Fluorogenic Substrates

The introduction of synthetic chromogenic substrates was a milestone in coagulation tests. The chromogenic assay allows a direct determination of the activity of an enzyme or protease inhibitor. They are very much related to the typical enzymatic assays in clinical chemistry and therefore easily automated. The principle briefly outlined is based on a peptide (2–4 amino acids) which is combined with a chromophore, e.g. *para*-nitroaniline (pNA) behind the cleavage site. The chromophore is coupled to the peptide by a peptide-like bond and shows an absorbance maximum at shorter wavelengths (380–405 nm) than the free chromophore. Proteases cleave the peptide, which leads to the liberation of the chromophore. Measured at an appropriate wavelength, the change of absorbance is a direct function of the activity of the enzyme.^{31,43,56,104,151}

In addition, fluorogenic substrates are also used. Commonly the fluorogenic group is 7-amino-4-methylcoumarin, which shows maximum fluorescence emission at 440 nm. Fluorogenic substrates allow the development of more sensitive assays than chromogenic ones, e.g. detection of trace amount of proteases. For both methods the peptide sequence of the substrate determines the specificity and sensitivity of the assays.^{31,43,56,104,151}

Assay Systems with Chromogenic Substrates

Usually the concentration of the chromogenic substrate is chosen so that a linear cleavage is obtained for at least the first 30–60 seconds, producing a kinetic assay. This means that the development of color is followed over time and should be linear, before the reaction rate decreases when the substrate is exhausted. Turbidity formation induced by fibrin generation during the assay can interfere with the photometric detection.^{31,43,56,104,151}

Immunochemical Methods

Generally, immunochemical tests detect the concentration of an analyte, and cannot easily discriminate between active and inactive forms. Using specific antibodies (monoclonal or polyclonal) the detection of reaction products such as activation peptides, enzyme-inhibitor complexes or breakdown products can provide information on ongoing activation of hemostasis.^{56,104,151}

The major techniques are the automated latex enhanced turbidimetric assays and the manual agglutination tests. The principle of both is basically the same: in the presence of antigen, antibody coated particles form agglutinates of large molecular size which show either a visible formation of large particles or a change in the turbidity of the suspension, which can be detected by photometry. A widely used method is the enzyme linked immunosorbent assay (ELISA) or its variant (sandwich technique).^{56,104,151}

COAGULATION SCREENING TESTS

Prothrombin Time

The prothrombin time (PT) was introduced as an assay for prothrombin in plasma samples before many of the other factors that participate in coagulation reactions were identified.¹²¹ It is a widely used screening test for the function of the extrinsic pathway. The test is sensitive for deficiencies of FVII, FX, FV, prothrombin (FII), and for fibrinogen (FI). The sensitivity for the latter two factors, FII and FI, is less pronounced than for the other factors. In man, the PT is often used for monitoring oral anticoagulants (vitamin K antagonists) and as a screening test to identify acquired or inherited deficiency or rarely occurrence of inhibitors.^{89,112,151}

The PT test is performed by adding tissue thromboplastin (or TF) and Ca^{2+} to citrated plasma and measuring the time for clot formation. It can be performed either manually by the tilt tube method or mechanically by use of a fibrometer or a photo-optical instrument. The PT reagent used in the testing provides the tissue thromboplastin and Ca^{2+} .^{89,151}

Currently three types of thromboplastin reagent are used, which can differ in several aspects such as factor sensitivity, lot to lot consistency, and absolute values of the clotting times. These types include: (1) recombinant PT reagents (recombinant tissue factor, Ca^{2+} , PL, buffer, stabilizers), (2) tissue thromboplastins (relatively crude extracts of thromboplastin-rich tissue, either from rabbit brain or human placenta, and Ca^{2+}), and (3) combined thromboplastin (tissue thromboplastin, e.g. from bovine brain, diluted into a fibrinogen fraction, usually adsorbed bovine plasma, and Ca^{2+}). The necessary Ca^{2+} is added to the reagent either at the time of manufacture or prior to testing.^{89,151}

The PT test can be performed as either a one-stage or a two-stage assay although the one-stage procedure is the most widely used and preferred. Thromboplastin reagent (0.2 mL) is warmed at 37°C then forcibly added to plasma (0.1 mL) which also has been heated to 37°C, and a timer is started. As soon as the clot forms indicating fibrin formation, the timing stops and the time is recorded to the nearest one-tenth of a second. The expected normal range for a PT is dependent on the type of reagent used.^{89,151}

The results of the prothrombin time assay are usually reported in seconds (Table 138.1). The international normalized ratio (INR) generally used in humans is seldom utilized in veterinary science, although it has been applied in some experimental studies.^{86,107}

In veterinary diagnostics, the higher activity of several coagulation factors involved in the pathways of hemostasis explored by the PT assay have led to suggestions to increase the results up to 10–15 seconds, similar to those results reported in humans.^{65,165} This could be done by diluting the plasma citrate samples (from 1:20 to 1:40)¹⁰⁵ or the thromboplastin reagent³¹ and/or by adding an established amount of human fibrinogen.¹⁰⁵ These procedures can increase the sensi-

TABLE 138.1 Ways of Reporting Prothrombin Time Values⁶⁷

Unit name	Definition	Notes
Seconds	Clotting time	Reagent, batch and instrument dependent
Prothrombin ratio (PR)	Ratio of clotting times of sample divided by clotting time of normal plasma	Reagent and instrument dependent; comparison between reagents cannot be possible
Percent activity (%)	Clotting time is related to a calibration curve obtained by dilution of normal plasma or with appropriate calibrators	Reagent dependent; technical error may occur; PT% values may differ between reagents; algorithm for calibration curve is not standardized
International Normalized Ratio (INR)	PR corrected by the ISI (International Sensitivity Index) value	Recommended for oral anticoagulation therapy control

tivity of the test in the dog,¹⁰⁵ in the cat¹⁰⁰ and in the horse.⁴¹

Prolongation of the PT test result can have several causes. It can be due to a reduced production of functional factor or to reduction of a combination of functional factors in both the extrinsic system and common pathway. Disorders where this can occur include liver failure, rodenticide intoxication by vitamin K antagonist, malabsorption, increased globulin levels (i.e. canine multiple myeloma or leishmaniasis), and congenital factor deficiency (factors VII, II, I).^{31,89,151}

A prolongation of PT results can also be induced by consumption or loss of plasma factor in disorders such as disseminated intravascular coagulation (DIC) or primary hyperfibrinolysis, and during thrombolytic therapy or chemotherapy.^{38,89,106} In addition, prolongation of the PT result can be induced by the presence of inhibitors or substances that interfere with fibrin polymerization, such as a heparin IV bolus. Finally, prolongation of the PT can derive from improper collection of blood (underfilled tubes) or can be seen in patients with elevated hematocrits, which alters the ratio between citrate and plasma (excess of citrate).^{73,89}

The degree of prolongation is inversely correlated with the factor plasma level, but a normal PT does not exclude a factor deficiency. It should be noted that the extrinsic pathway is less sensitive to reduced factor levels in comparison to both common and intrinsic pathways.^{89,151}

Shortening of the PT can also occur, but PT results shorter than the reference ranges have not been consistently correlated with any clinical situation except hyperfibrinogenemia.⁷⁴ It should also be noted that lipemic and icteric samples can shorten the PT results in dogs.¹⁰⁹

Activated Partial Thromboplastin Time

The activated partial thromboplastin time (aPTT, sometimes abbreviated as PTT) is a screening test for the intrinsic system and common pathway. The name "partial thromboplastin" means that the reagent contains PL but no TF, in this way activating only the intrinsic system. "Activated" means that reagents contain substances that can accelerate the coagulation reaction and shorten the time to produce the fibrin clot.^{90,112}

The aPTT assay is performed by adding to platelet-poor plasma (PPP) factor XII activator, PL, and Ca²⁺ (as calcium chloride, 20 or 25 mM). Factors I, II, V, VIII, IX, X, XI, XII, prekallikrein (Fletcher factor) and high molecular weight kininogen (HMWK) are measured. An abnormal aPTT result might indicate the presence of an acquired inhibitor or a deficiency in any of the coagulation factors except FVII and FXIII.^{90,112,151}

The aPTT reagents are even more diverse and unstandardized than PT reagents. Commonly used activators are ellagic acid, kaolin, micronized silica and celite. All of these are used in aPTT reagents and serve the same function of activating the clotting mechanism. Phospholipids are platelet substitutes and accelerate the reactions involved. Sources of PL are rabbit brain, cephalin (dehydrated rabbit brain), bovine brain, and soybean. Usually only mixtures with different types of PL, including the negatively charged phosphatidyl serine, are used. It is the combination of contact activator, PL, calcium chloride molarity, ionic strength, buffer systems, and the stabilizers of the reagents, which determines the reagent properties.^{90,112,151}

A suspension of activator and partial thromboplastin (aPTT reagent) (0.1 mL) is warmed at 37°C for 2–5 minutes together with 0.1 mL of a citrated plasma sample. A timer is started as 0.1 mL of calcium chloride solution (37°C, 0.025 M) is added. As soon as the clot forms indicating fibrin formation, the timer is stopped and the time is recorded to the nearest one-tenth of a second.^{90,112}

In animal testing, the normal values should range from 10 to 20 seconds, taking into account the different concentration and activity of coagulation factors involved in aPTT, which are known to be species-specific.^{65,79,165}

The normal range of the aPTT is strongly reagent and instrument dependent, but a significant role is played by the pre-analytical phase. Each laboratory should determine its own normal reference ranges (sometimes for each new lot of reagent).^{90,112,151}

Prolongation of the test result can have several pathogenic mechanisms. It can be caused by a reduced production of each factor or by a combination of factors in the intrinsic and common pathway. The list of disorders includes liver failure, rodenticide intoxication by vitamin K antagonist, malabsorption, increased globulin levels (i.e. canine multiple myeloma or leishmaniasis), congenital factor deficiency (factor VIII, IX, XI, XII,

prekallikrein, HMWK, X, V, II, I), and congenital deficiency of von Willebrand factor (VWF) (aPTT can be slightly prolonged due to the reduced half life of factor VIII).^{90,106,151}

Prolongation of aPTT results can also be induced by consumption or loss of plasma factors seen in disorders such as DIC or primary hyperfibrinolysis, or with thrombolytic therapy or chemotherapy.^{38,90,106,151}

In addition, a prolongation of aPTT result can be induced by the presence of inhibitors or substances that interfere with fibrin polymerization such as heparin, heparin-like substances, lupus anticoagulant, and autoantibody against one of the coagulation factors involved in the aPTT.^{90,151}

Finally, prolongation of aPTT, similar to PT, can result from improper collection of blood (underfilled tubes) or from patients with an elevated hematocrit, which alters the ratio between citrate and plasma (excess of citrate).^{73,117}

Shortened aPTT results can occur, and may be related to the presence of activated coagulation factors. Attention should be paid to accurate and appropriate blood sampling.^{90,112} Indeed, highly hemolytic canine samples can shorten the aPTT results.¹⁰⁹

Reagents used for aPTT show variable factor sensitivity, especially for FVIII and FIX deficiency. Sensitivity depends strongly on the accuracy of the selected cut-off values for interpretation of normal ranges. Clinically significant deficiencies with levels below 30% are usually identified.^{94,95}

Thrombin Time

The thrombin time (TT or thrombin clotting time) is a simple screening test for both the fibrinogen conversion to fibrin and fibrin polymerization. The TT is inversely proportional to the fibrinogen level. This test can detect dysfibrinogenemia, heparin and excess of fibrin/fibrinogen degradation products (FDPs).^{40,151}

This test reflects two different reactions: the cleavage by thrombin of fibrinopeptides from fibrinogen to produce fibrin monomers and the polymerization of fibrin monomers into the fibrin polymer, which is identified as a fibrin clot.^{40,151}

The TT test is performed by adding a relatively low to moderate concentration of thrombin (bovine or human) into citrated plasma. Thrombin cleaves fibrinogen, releasing fibrinopeptide A (FPA) and B (FPB), and converting fibrinogen into fibrin clot. The clotting time is related to the fibrinogen concentration and quality and to the presence of AT. The test is strongly method-dependent.^{40,112,151}

The prolongation of the test can be caused by reduced levels of fibrinogen, presence of heparin or heparin-like substances, elevated FDPs, and monoclonal paraprotein, dysfibrinogenemia, and hyperfibrinogenemia.^{28,40,106,151}

Shortened TT results are rare. Improper sample collection/manipulation and storage need to be considered; indeed lipemic and hemolytic samples can shorten the TT results in dogs.¹⁰⁹

Reptilase® Time

The Reptilase® time (batroxobin) test is based on the use of a thrombin-like snake venom protease which is able to clot fibrinogen. However, in contrast to thrombin, batroxobin cleaves only FPA and has no activity directed against other thrombin substrates such as FV, FVIII, etc. This test is not inhibited by AT and heparin and can be used to investigate fibrin polymerization in the presence of heparin. The assay is not commonly used in current veterinary diagnostics.¹⁵¹ The Reptilase® time is prolonged in afibrinogenemia, severe fibrinogen deficiency, dysfibrinogenemia and in the presence of relatively high levels of FDPs.¹⁵¹

DETERMINATION OF FIBRINOGEN AND INDIVIDUAL COAGULATION FACTORS

Fibrinogen

Several techniques to assay fibrinogen are available as functional or immunochemical methods, including total clottable fibrinogen, thrombin-initiated clotting rate, thrombin-initiated turbidimetry or light scattering, salt precipitation and fibrinogen antigen measurement.^{12,43,113,151}

The most commonly used assay is the thrombin-initiated clotting rate, modified by Clauss.²⁵ This method is based on the rate of clot formation in diluted citrated plasma following the addition of a high concentration of thrombin solution. In this way high concentrations of thrombin minimize interference by heparin therapy, while diluted citrated plasma reduces interference by high levels of FDPs, as happens in DIC. However, high levels of FDPs can interfere and lead to falsely low values for fibrinogen, as documented in dogs.^{25,97,103,113}

Another assay is based on the increase of turbidity during the PT test, which is directly proportional to the concentration of clottable fibrinogen and could be detected by nephelometry or turbidimetric end-point detection. Therefore the PT derived fibrinogen assay correlates well with the Clauss method, with the exception of patients with elevated levels of FDPs. Here, the PT derived fibrinogen is usually higher than the Clauss assay, but is probably more accurate.^{12,67,151}

A kinetic assay for fibrinogen is available which employs batroxobin, which generates a pseudo-linear increase of turbidity. This method has a wide range, an excellent precision and can be easily be adapted to automated coagulometers or clinical analyzers.^{12,67,151}

The antigen assay for fibrinogen, belonging to the immunochemical methods, measures the quantity of fibrinogen and does not discriminate between clottable fibrinogen and inactive or degraded forms. This assay may produce significantly higher values of fibrinogen in the presence of high concentrations of FDPs. Unfortunately, it appears that this assay will have limited use in veterinary medicine until species-specific antibodies are available.^{111,122}

The estimation of fibrinogen by the heat-denaturation method correlates well with chronometric proce-

dures, except during acute-phase reactions.^{19,92} Some studies comparing several assay methods have been done in dogs.¹⁰²

Hypofibrinogenemia can be observed in DIC, hyperfibrinolytic syndrome, liver disease, inherited hypofibrinogenemia, dysfibrinogenemia, multiple myeloma, and with thrombolytic therapy or with the presence of monoclonal paraproteins. Hyperfibrinogenemia can be seen in acute phase responses, with glucocorticoid administration, and with pregnancy.^{12,151}

Sources of analytical error can be: over- or underfilling of collection tubes, lack of citrate volume correction for high hematocrit, use of the wrong anticoagulant and heparin contamination.¹²

Coagulation Factor Assays: FII, FV, FVII, FVIII, FIX, FX, FXI, and FXII

Clotting Assays

Specific clotting factor assays are generally used to diagnose inherited or acquired hemorrhagic disorder, or to explain PT or aPTT prolongation. Although specific chromogenic assays for many of the individual coagulation factors are available, clotting assays are the preferred methods for coagulation factor assays. With this principle the activity of several coagulation factors can be determined in a modified PT (for FVII, FX, FV, FII) or aPTT (for FXII, FXI, FIX, FVIII, and FV also) test, in combination with a factor deficient plasma reagent. A factor deficient or substrate plasma contains all but the factor to be assayed. The most important quality criteria for the factor deficient plasma reagents (either immunoabsorbed or congenital) are a very low or undetectable level of the factor which is to be assayed, high activity of all other factors and a steep calibration curve (prepared with pooled plasma of the same species where the test is applied) which allows a good discrimination between normal and abnormal.^{31,58}

In veterinary diagnostics, this type of assay system assumes that there is sufficient structural homology between the human proteins and those of other species, so that they can react interchangeably in the thrombin-generating pathway. Consequently, human plasma reagents are generally used. However, this type of assay is not necessarily reliable when the absolute activity for a non-human species is being determined. For example, striking differences in structural homology have been pointed out for sheep and cattle plasma.^{43,65}

The plasma under investigation is diluted (1:5 or 1:10) into the deficient plasma. The degree of correction of the very long clotting time of the deficient plasma is proportional to the activity of the specific factor in the sample. The assay is calibrated by running dilutions of a standard or calibrator, which for animal studies is the pooled plasma. The calibration curve gives a straight line over a wide range in a double logarithmic plot.^{58,151}

Factor assays that depend on a clot as the end-point of reaction are inherently quite variable, with inter-assay coefficient of variation in the range 10–15%. Not

all combinations of deficient plasma and PT and aPTT reagent work equally well. The quality of the deficient plasma and the factor sensitivity of the reagent play significant roles.^{58,151}

The reference interval for each factor level is expressed as percent of normal pooled plasma concentration. By definition, normal plasma contains 100% (1 unit/mL) of each factor. The reference interval is approximately 60–140%.^{58,151}

It has been pointed out that the type of commercial prothrombin time reagents and methods used are critical in equine or canine plasma evaluation for FII, FV, FVII, and FX deficiencies.^{43,96,97,99,101} Conversely, most commercial aPTT test kits are sensitive as screening tests for factor VIII and IX deficiencies in canine plasma.^{94,95}

Assays for Inhibitors

Because acquired causes of factor deficiencies are generally more common than hereditary causes, a patient found to have a factor deficiency should be evaluated for possible acquired etiologies. Differentiating between true deficiencies and inhibitor occurrence is the second step to correctly approach these patients. A description of inhibitors and their testing is provided below.⁶⁷

Lupus Anticoagulant Historically these inhibitors were noted in patients with systemic lupus erythematosus (SLE), but later it was evident that they were not restricted to patients with SLE, and that they do not have an anticoagulant effect in vivo. Despite aPTT prolongation, patients with this inhibitor had increased risk for various thrombotic sequelae. The evidence that the mechanism of disease was due to antibody that binds to the phospholipid in the aPTT reagent caused the antibody to be renamed “antiphospholipid antibody.” Presence of an inhibitor, such as lupus anticoagulant, has been rarely demonstrated in veterinary species but it is likely that its occurrence is underestimated.¹³⁹

The presence of an inhibitor, such as lupus anticoagulant, can cause artifactual decreases in the in vitro factor level. Therefore, the laboratory should perform factor assays at multiple dilutions (three minimum). At higher dilutions, the inhibitor interference will decrease due to dilution of the inhibitor.^{59,151}

This assay is done by mixing patient's plasma and an equal volume of normal plasma, noting aPTT results immediately and after 1 hour of incubation at 37°C. A significant factor deficiency is suggested if the test normalizes. If the test remains altered or increased, the inhibitory substance is confirmed. Partial correction is not uncommon in human samples and this presentation can be difficult to interpret even with more detailed studies. To confirm that reduction of phospholipid is the cause of aPTT prolongation, additional study is needed. In this case a modified aPTT or PT or Russell viper venom time with dilute phospholipid is used. A suitable control plasma is either tested in parallel or

reference intervals are determined with plasma from healthy donors.^{59,151}

Factor VIII Acquired Inhibitor Some hemophilic patients who have been transfused with products containing FVIII have developed antibodies against FVIII, which inactivates the transfused factor. The predisposition to inhibitor development is genetically based. This happens in humans and rarely in dogs.^{44,141}

A quantitative determination of the inhibitor titer can be made in specific clotting assays such as the “Bethesda assay” or the “Oxford” or “Nijmegen” assay for FVIII or for other coagulation factors. In these assays normal plasma is incubated with different dilutions of the sample or a normal control. The inhibitors inactivate the FVIII of the normal plasma after an incubation step of 2 hours at 37°C. The residual activity in the samples is determined and the inhibitor titer is calculated, i.e. a titer of 1.0 is defined as the inhibition of 50% of FVIII in the normal plasma.^{4,151}

Factor IX and XI Acquired Inhibitors Repeated blood product transfusions can cause acquired inhibitors of FIX and FXI. These are very rare situations in veterinary medicine, with one case report in a cat described.³⁷

Monoclonal Paraproteins Multiple myeloma is a well-described disorder in veterinary medicine. It is a clonal proliferation of plasma cells or their precursor in bone marrow. These cells are characterized by the production of monoclonal or more rarely biclonal gammaglobulins that can interfere with fibrin polymerization.¹²⁸

Actually, the mechanisms by which multiple myeloma cause hemostatic alterations and bleeding tendency are numerous: interference between VWF and glycoprotein Ib binding, decreased production of coagulation factors, production of both lupus anticoagulant and heparin like substances, specific inhibitor of factor VIII, and thrombopathia.^{45,66,140}

Heparin Canine mast cell neoplasia can produce a hemorrhagic paraneoplastic syndrome due to the production of heparin by neoplastic cells. Both TT and aPTT are prolonged and canine patients show a hemorrhagic tendency and dehiscence of healing wounds.⁵⁷

Chromogenic Assays

For the evaluation of several coagulation factors, chromogenic assays may be superior (and cheaper) when better resolution in the high range is required. The high sensitivity of the chromogenic methods allows a higher sample dilution, which eliminates several problems connected with inhibitors, which might interfere with the clot-based methods.^{43,151}

The determination of FX is one of the most traditional chromogenic substrate methods. The activation of FX in the sample made with the FX activating enzyme from Russell viper venom is directly detected by a chromogenic substrate such as Pefachrome® Xa.^{43,151}

The determination of FVIII by chromogenic methods is the most widely used assay, especially in human medicine. Indeed, it is the method of choice during the monitoring of substitution therapy using recombinant or monoclonal purified FVIII concentrates. As FVIII is not an enzyme, it catalyzes the activation of FX by FIXa in the presence of PL and Ca²⁺. The amount of FXa which is generated is directly proportional to the activity of FVIII in the sample. Methodological factors can lead to systematic differences between the clotting assay and the chromogenic method, but can be overcome by careful standardization of the test systems and by use of appropriate calibrators.⁵

A chromogenic assay for FIX has been described in which FIXa, previously activated by FXIa, activates FX in the presence of a saturating amount of FVIIIa. The activity of generated FXa is directly proportional to the activity of FIX.⁴⁷

The determination of FVII should be performed in a coupled assay as FVIIa is a poor “amidase” and does not hydrolyze synthetic substrates effectively enough for a direct assay. In the sample FVII is activated with TF, PL, and Ca²⁺. The complex FVIIa-TF activates the FX in the reagent. The FXa can be detected with a chromogenic substrate, such as Pefachrome® Xa.¹⁵¹

The chromogenic assays have been shown to be effective in estimating FVII, FVIII, FIX and FX in horses and in a few other species.^{43,143}

Finally, immunoassays (antigen assays) are commercially available in man for FVII, FVIII, FIX, and FX. Unfortunately, it appears that these assays will have limited use in veterinary medicine until species-specific antibodies are available.^{111,122}

It should be pointed out that factor deficiencies can be quantitative or qualitative. In quantitative disorders, the factor level determined by routine PT- or aPTT-based methods (functional activity assays) is similar to the result obtained by immunological (antigen) assays. In qualitative disorders, the PT- or aPTT-based (functional) assay result is decreased, but the antigen level is normal or significantly higher than the functional level, indicating the presence of a dysfunctional protein.¹⁵¹

Determination of von Willebrand Factor

The analysis of VWF is quite complex as several subtypes of this disease are known (Chapter 81). The most often performed assays in veterinary science include:

1. determination of the concentration of VWF antigen (Ag), by means of immuno-turbidimetric assay and ELISA^{3,9,10,62,78,131}
2. VWF subunit pattern analysis, by means of agarose gel electrophoresis and immunoblot⁶⁷
3. determination of activity using agglutination of fixed or fresh platelets induced by ristocetin, botrocetin, or collagen, and measuring the agglutination that has occurred by an aggregometer.⁶⁴

Alternatively, immunoassays have been designed to assess VWF function. The collagen-binding assay is

an ELISA in which collagen is the antigen. If VWF is functional, it binds to collagen and is subsequently detected.¹⁵¹

Repeated testing is often required, because both VWF and FVIII become elevated above baseline during acute phase reactions, including even minor illnesses, injury, stress, or pregnancy. An elevation of a low or borderline value for VWF into the normal range during any of these conditions often masks the diagnosis of VWD. It should be noted that when VWF is markedly decreased, the FVIII level can also become very low, which prolongs the aPTT. In most VWD patients, the disease is mild or moderate and the aPTT is therefore normal.^{88,110}

Finally, it should be pointed out that currently diagnosis of VWD can be performed in many dog breeds by means of molecular testing.^{17,69,124}

TESTS FOR THROMBOPHILIA

Antithrombin

Antithrombin (AT) (previously antithrombin III or ATIII) seems to be one of the most important physiologic protease inhibitors in plasma involved in the regulation of blood coagulation. Antithrombin is a protein produced mainly by hepatocytes and partially by endothelial cells. Its main function is to bind to thrombin, forming a 1:1 stoichiometric complex and preventing the conversion of fibrinogen to fibrin. In addition, the AT action is similar against other activated factors (FXIIa, FXIa, FIXa, FXa and partially FVIIa). This inhibitory effect of AT is catalyzed by heparin and by proteoglycans such as heparin sulfate (HS), which may be present in high concentrations locally at the endothelial surface.^{52,125} Due to its cofactor role in heparin therapy, AT is a frequently ordered assay in thrombophilia, DIC and during heparin therapy.^{16,48,120,123,142} In veterinary clinical practice, chromogenic assays are primarily used to measure AT, as they show a low interassay coefficient of variation.^{36,152} In the chromogenic assay AT is determined using FXa or thrombin in the presence of a saturating concentration of heparin (heparin cofactor method). This is the preferred method of testing as assays using thrombin are available but FXa is not inhibited by heparin cofactor II and is more stable. The general principle is based on an inhibition phase in which FXa excess is reacted with AT in the presence of heparin to give an inactive FXa-AT complex plus a residual FXa. The FXa residual reacts with the chromogenic substrate to give a color which can be measured spectrophotometrically.^{35,60,151} The values of AT are reported as percent of activity compared to either a species-specific or human plasma pool considered to have 100% activity. Consequently, reference and reported values vary with the species used for the reference pool in relationship to species differences in AT activity.^{16,48,120,123}

Inherited deficiencies of AT, a common cause of spontaneous or recurrent venous thromboembolic

events in humans, are not reported in veterinary species. In clinical veterinary practice acquired plasma variations of AT are more frequently observed (see Chapter 87).

Protein C

Protein C (PC) is a vitamin K-dependent plasma protein synthesized in the liver. The binding of thrombin to thrombomodulin (TM) converts the bound thrombin from a procoagulant enzyme to an anticoagulant enzyme that converts the PC zymogen to the anticoagulant serine protease renamed "activated protein C" (APC). This surface-dependent reaction is enhanced by the endothelial cell protein C receptor that binds PC. Protein C testing may be of value in the determination of pre-thrombotic or hypercoagulable conditions.^{33,34,154}

The currently performed methods are either functional or immunochemical. The functional assays are either clotting or chromogenic substrate based assays.^{26,149,151}

The PC clotting assay is performed in a way similar to the assays for the individual coagulation factors. The sample is pre-diluted into PC deficient plasma which is prepared by immunoabsorption. In the activation phase, the PC in the sample is activated by Protac® (a snake venom based PC activator from *Agkistrodon contortrix*). The inhibiting effect of PC is detected via the aPTT. The prolongation of the clotting time (caused by inactivation of FV and FVIII) is proportional to the activity of PC in the patient plasma. This assay can easily be automated and the quality of PC deficient plasma is important.^{26,149,151}

In the dog it has been demonstrated that Protac® does not stimulate the production of activated PC activity in canine plasma detectable by clotting assays. Molecular differences in the canine PC molecule are hypothesized to prevent formation of the stoichiometric complex of venom extract, aPTT reagent, and canine protein thought to be essential for the PC-activating function of the Protac®.⁶³

The PC chromogenic assay is based on the activation of PC by Protac® to give APC, which reacts with the chromogenic substrate to give a color by means of pNA that can be measured spectrophotometrically. This assay is very simple, specific, easy to automate and shows an excellent precision.^{26,149,151}

The immunochemical determination of PC by means of automated turbidimetric agglutination tests and ELISA is less important than the functional method.^{26,149,151} An antigenic assay has been developed in the horse which shows poor correlation with the clotting method. The values are reported as percent of activity or antigen relative to a reference plasma pool considered to have 100% activity or antigen.^{6,157,158}

Activated protein C resistance (APCR), a reduced anticoagulant response of a plasma sample to APC, is an important risk factor for thrombosis in man.^{67,149,151} Activated protein C resistance is assayed via a modified aPTT in the presence and absence of APC. Prothrombin time and snake venom-based tests have also been

described. Unfortunately, no literature is available in veterinary science about the use of APCR testing.^{67,151,154}

Protein S

Protein S is one of the vitamin K-dependent coagulation proteins and its main function is to enhance the anticoagulant activity of APC as cofactor. In human medicine, deficiencies of PS are associated with inherited thrombotic disease.^{26,149,151} In veterinary science, no references are available on clinical applications for the assay. A study examining the effects of bovine protein S and bovine APC on fibrinolysis in human plasma has been published.¹⁵⁴

TESTS FOR THE MONITORING OF ANTICOAGULANTS

Anticoagulant drugs are widely used for prophylaxis and treatment of thromboembolic disorders and in DIC. Strict monitoring and dose adjustment according to the results of appropriate tests are necessary in order to prevent bleeding or thrombosis. Monitoring can either be performed in the laboratory or by near patient testing (NPT) with appropriate devices. Testing in the laboratory is performed on citrated plasma whereas NPT can be performed directly on non-anticoagulated whole blood or with citrate added.

Activated Clotting Time

The ACT or activated coagulation time is a “bedside” clotting or a point-of-care test. It is similar to the principle of aPTT with the difference that the ACT is activated with a contact activator without the addition of PL and Ca^{2+} .^{67,91,151}

Activated clotting time testing is very simple, using whole blood that immediately after collection is transferred to a special tube containing an activator of coagulation, such as celite (diatomaceous earth), kaolin, or glass particles. These activate the intrinsic pathway of coagulation, causing the blood to clot. The tubes require accurate mixing and the specimen cannot be stored.^{20,46,49,91,163}

For methods that use cartridges rather than tubes, whole blood may be collected into a plastic syringe or tube and then immediately transferred into the cartridge.^{55,91} In the test tube method, a visual inspection can be performed to detect clot formation, which can lead to inaccuracy. Alternatively, a specialized coagulation analyzer, which detects the clot formation by the mobility of a magnet inside the blood test tube, can be used. Mechanical, electrical or light devices, or cartridge methods can detect clot formation.^{91,150}

The ACT is less precise than the aPTT, and lacks high correlation with the aPTT or with heparin anti-FXa levels. The ACT is influenced by a number of variables, including platelet count, platelet function, lupus anticoagulants, factor deficiency, room temperature, hypothermia, and hemodilution. The various methods are

not standardized, and therefore, results obtained are not interchangeable.⁹¹

Activated Partial Thromboplastin Time

The aPTT assay described in detail earlier can be useful for the monitoring of heparin in the lower concentration range. However, as a screening test, aPTT is not only influenced by anticoagulant concentration but also by a variety of other factors; for example, the unfractionated heparin (UFH) assessment shows a pronounced reagent dependency.^{18,98}

Heparin Assays

Synthetic substrate based assays are widely used for the specific determination of heparin, especially when low molecular weight heparin (LMWH) is used.^{67,151} Heparin is determined via its accelerating function in the inhibition of FXa (anti-FXa activity) or thrombin by AT. The stimulation of AT inhibition of FXa by LMWH or degraded forms of UFH favors the pathway via FXa. FXa has also the practical advantage that is a relatively stable enzyme. The test is based on two principal steps:^{67,151} the inhibition phase where an excess of FXa plus AT in the presence of heparin in the sample gives FX-AT inactive forms, and the FXa residual molecules detection phase where FXa residual molecules react with a chromogenic substrate and give a color via pNA that is detected spectrophotometrically.

The test can either be arranged as a kinetic or end-point version. There are several modifications of this general principle. Some tests use a saturating concentration of AT in the reagent; others are based on the endogenous AT in the sample. Whereas the first modification determines the “total heparin” the latter determines the AT-heparin complex.^{67,151}

Chromogenic heparin assays can be easily automated and are especially useful for the monitoring of LMWH for which no routine clotting assay (such as aPTT) is available. In addition, the assay shows no interference by FDPs, aprotinin or elevated FVIII values, which have a strong impact on the aPTT or ACT tests.^{67,151}

The calibration of heparin assays can vary since several manufacturers recommend calibration with the same brand of heparin used for therapy. The measuring range for most commercial assays is from 0 to 1.0 anti-FXa U/mL, while the therapeutic range is usually between 0.3 and 0.7 anti-FXa U/mL. Extensive studies have been conducted in canine and feline medicine assaying the several commercial kits available.^{18,30,98,127}

FUNCTIONAL TESTS FOR FIBRINOLYSIS

Chromogenic substrate tests brought fibrinolysis function tests into practical laboratory settings. Previously, fibrin plate lysis assays, the euglobulin lysis time and similar complicated and time-consuming tests were used by only specialized laboratories.¹³ In man, tests are available for measurement of

plasminogen, alpha-2-antiplasmin (α 2-AP), plasminogen activator inhibitor 1 (PAI-1), and tissue plasminogen activator (t-PA).^{13,145,146,147,151} Unfortunately, these assays are costly and used primarily for research purposes in veterinary medicine, with limited veterinary practice application.^{75,76}

Plasminogen

Plasminogen is the inactive precursor of the enzyme plasmin, which is the primary catalyst of fibrin degradation. The assay for plasminogen utilizes the interaction of plasminogen with streptokinase, a bacterial protein. In veterinary science, urokinase instead of streptokinase is used for both dogs and horses. The complex of plasminogen and urokinase has enzymatic activity and hydrolyses the chromogenic substrate, releasing a colored-compound. This complex is not inhibited by alpha-2-macroglobulin and the fast acting α 2-AP. An assay which would simply convert plasminogen directly into plasmin is not possible in plasma, because free plasmin is immediately neutralized by α 2-AP.^{36,75,145,156}

For the plasminogen assay, aliquots of standard plasma, control plasma, and the patient's test plasma are dispensed into reaction tubes. Streptokinase (urokinase in dogs and horses) is added in excess, so that virtually all the plasminogen in the sample is in the complex form with plasmin-like activity. Following a short incubation period, the appropriate chromogenic substrate is added and the reaction is allowed to proceed. After 0.5–5 minutes the reaction is stopped with acetic acid and the color is assessed at the appropriate wavelength.^{67,145,146,151,156}

Acquired abnormalities of plasminogen are encountered far more commonly than inherited deficiencies. A low concentration of plasminogen can be caused by increased consumption (DIC, thrombolytic therapy) and decreased production (liver failure).^{67,145,146,151}

Alpha-2-Antiplasmin

Alpha-2-antiplasmin is the primary plasmin inhibitor in plasma and thus an important regulator of fibrinolysis. It is synthesized by hepatocytes, although the kidney may be another contributing source. Evaluation of the concentration of α 2-AP may be useful in patients with thrombophilia, unexplained bleeding and DIC.⁸⁷

Chromogenic assays for plasma α 2-AP have replaced other test methods (esterolytic, fibrin plate lysis) previously employed. Immunologic methods for the measurement of α 2-AP are also available, but it is preferable to assess enzymatic activity rather than the total mass, at least as an initial assay. Many inhibitors in plasma can react with plasmin, but α 2-AP is the only extremely fast acting inhibitor. Due to the rapid inactivation of plasmin by α 2-AP, the inhibition and detection phase can be started almost simultaneously, which helps to improve specificity against slower acting plasmin inhibitors in the plasma. The test includes an inhibition phase where an excess of plasmin links to α 2-AP in the

sample. The residual plasmin left reacts with the chromogenic substrate, releasing a colored-compound.^{75,145}

Acquired abnormalities of α 2-AP are encountered far more commonly than inherited deficiency. Low concentration of α 2-AP can be caused by increased consumption (DIC, thrombolytic therapy) and decreased production (liver failure).^{67,145,151}

Plasminogen Activators

There are two types of plasminogen activators: tissue-type plasminogen activator (t-PA) and urokinase-like plasminogen activator (u-PA). Both types are serine proteases that have high specific activity in converting plasminogen to plasmin.¹⁴⁶

Though t-PA is a relatively good amidase which can be easily determined in purified preparations with specific chromogenic substrates, its low concentration in plasma requires an indirect approach. One molecule of t-PA can activate multiple molecules of plasminogen and a massive amplification is obtained. Therefore the determination of the low concentrations of t-PA, which are usually found in plasma, is possible in an amplification system via plasminogen and catalyzed by soluble fibrin (in the form of cyanogen bromide cleaved fibrinogen).^{146,151}

Increased levels of t-PA can be caused by liver cirrhosis, acute promyelocyte leukemia, and in the reperfusion phase of liver transplantation (both only in humans). Decreased levels of t-PA can be caused by trauma, surgery, pregnancy, and thrombotic thrombocytopenic purpura.^{146,151}

Plasminogen Activator Inhibitor 1

Plasminogen activator inhibitor 1 is produced by endothelial cells, platelets and hepatocytes. In plasma, PAI-1 circulates in complex with vitronectin, which may protect it from inactivation. It is the primary physiologic inhibitor of plasminogen activation in blood, targeting both u-PA and t-PA. The measurement of its plasma level therefore provides important information as to the balance between profibrinolytic and antifibrinolytic factors. Elevations of PAI-1 may be associated with a predisposition to thrombosis, whereas deficiency of PAI-1 on an inherited basis has been associated with a bleeding disorder.⁷⁷

The determination of PAI-1 is technically complicated. In blood PAI-1 circulates in three major forms: inactive (20%), or "latent", PAI-1; active PAI-1 (60%); and PAI-1 in complex (20%) with t-PA. PAI-1 can be assayed using both functional and immunologic methods. For both methods, sample collection is a crucial point.^{72,77,151}

A simple direct inactivation assay via t-PA or u-PA is not sensitive enough for plasma because the concentration of PAI-1 is quite low. Typically an amplification step which utilizes the u-PA or t-PA mediated activation of plasminogen is used. The generated amount of plasmin, which is inversely proportional to the PAI-1 activity in the sample, is detected with a plasmin spe-

cific chromogenic substrate. Another difficulty is the direct or indirect interference by α 2-AP or alpha-2-macroglobulin. These inhibitors can be inactivated by acidification, oxidation or snake venom enzymes. When t-PA is used, soluble fibrin or cyanogen bromide derived fibrinogen peptides (as part of the reagent) are required as a stimulator for the amplification step. The u-PA based methods are technically somewhat less complicated and can be adapted to fully automated analyzers.^{72,75,146,147}

TESTS FOR HEMOSTASIS ACTIVATION

The majority of tests for the detection of hemostasis activation are based upon immunochemical methods. Monoclonal antibodies, which are able to discriminate between activated, non-activated, inhibitor-bound or inactivated forms of analytes are very useful. Using assays such as ELISA, automated fluorescence immunoassay or turbidimetry, activation markers have found increased applications in many referral laboratories. Manual latex agglutination assays are also used with reliability in veterinary practices.

D-Dimer

D-Dimer is a neoantigen formed when thrombin initiates the transformation of fibrinogen to fibrin; it is derived from plasmin digestion of cross-linked fibrin.^{22,134,137}

This analyte is determined with a monoclonal antibody-based assay. Turbidimetry, manual latex agglutination, fluorescence immunoassay, and ELISA are the available methods. The manual agglutination method is less sensitive than automated methods or ELISA and may show a high degree of variability. Since D-dimer is not a standardized analyte, different assays may show discrepant results depending on the specificity of the antibodies and the calibration material which is used. Results are reported as $\mu\text{g/mL}$ or ng/mL or $\mu\text{g/L}$.^{22,134,137}

Assays based on latex agglutination can estimate semiquantitatively^{29,116,137} or qualitatively^{50,51} the D-dimer concentration in dogs. The immunoturbidimetric assay is currently the gold standard for D-dimer concentration assessment in dogs and cats, and can use citrated plasma that has been stored for up to 1 month.^{16,22,39,115,134} In the horse, a comparative study for the assessment of several assays for D-dimer concentration has been performed, establishing the validated use of latex agglutination methods over the immunofiltration card test.¹³⁸

Fibrin/Fibrinogen Degradation Products

The determination of FDPs is a valuable tool for the detection of fibrinolytic activation, allowing an estimation of the relative proportion of fibrinolysis and fibrinogen breakdown, especially for clinical investigations in dogs, cats and horses.^{11,21,136,138}

Traditional methods with polyclonal antibodies against FDPs initially required special blood collection tubes, which contained either thrombin or thrombin-like snake venom (*Botrox atrox*) for clotting the blood. The assay can now be performed on citrated plasma samples, which are easier to collect and give fewer false positive results.^{21,136,138}

In the assay, patient plasma is mixed with latex particles, which are coated with monoclonal anti-FDP antibodies. If FDPs are present in the plasma, the latex particles agglutinate as FDPs bind to the antibodies on the particles. The technician can detect these large agglutinated clumps visually. Various dilutions of patient plasma can be tested to provide an estimation of the FDPs titer in a semiquantitative result format. The results are usually reported in $\mu\text{g/mL}$, ng/mL or $\mu\text{g/L}$.^{11,21,136,138}

Thrombin Anti-Thrombin Complex

The thrombin anti-thrombin (TAT) complex is an indicator of the inhibition of thrombin by its natural inhibitor AT. In the ELISA for TAT, a thrombin specific capture antibody binds the TAT complex. After a washing step, a second antibody detects the AT moiety of the complex in a color reaction. The values are reported in $\mu\text{g/L}$ or ng/mL .¹⁵¹ The assay has been applied in horses^{142,144,155}, dogs⁸⁴ and in other laboratory animal species.¹²²

Prothrombin Fragment F 1+2

Prothrombin fragment F 1+2 (F 1+2) is released from prothrombin after activation by the prothrombinase complex. While TAT represents a group of activation markers, characterized by complex formation between an active enzyme and an inhibitor, F 1+2 detects activation by prothrombinase. It represents the FXa activity *in vivo*.^{7,122,151}

Fibrinopeptide A

The FPA is a circulating amino acid peptide released from fibrinogen upon cleavage by thrombin.¹⁴⁸ The first generation of assays was based on separation of FPA and fibrinogen or larger FDPs by adsorption to bentonite. The detection of FPA in the bentonite supernatant was made with polyclonal antibodies directed against fibrinogen. Since then, monoclonal antibodies against FPA have been developed and the detection is based upon ELISA.^{122,151}

Soluble Fibrin

Like FPA, the determination of soluble fibrin (SF) or fibrin monomers (FM) is also direct evidence for thrombin formation *in vivo*. While FPA or TAT are cleared rapidly from the circulation, SF is present in plasma for several hours. The determination of SF with ELISA makes use of specific monoclonal antibodies

directed against the N-terminus in fibrin, formed when fibrinogen is cleaved by thrombin. Sample pretreatment using a chaotropic agent (a substance which disrupts the three dimensional structure in protein macromolecules) may be used. Generally, the results for SF and F 1+2 tend to be very similar.^{53,80,151}

The determination of SF can be performed by chromogenic substrate based assays, which use the potentiating effect of SF on the activation of plasminogen by t-PA. The activity of plasmin that is generated is proportional to the concentration of soluble fibrin. FDPs may also stimulate this reaction. Care has to be taken since FM may bind to plastic surfaces of the cuvettes.¹⁵¹ Recently, a latex immunoturbidimetric assay for SF has been validated in man.⁵³

THROMBOELASTOGRAPHY

The acknowledgement of the cell-based model of hemostasis (Chapter 84) has forced a paradigm shift in the theoretical perception of the biochemistry of hemostasis. It has challenged the traditional view of the intrinsic and extrinsic pathways in the cascade model of coagulation as accurate models of the complex physiology and pathophysiology of hemostasis in areas other than specific factor deficiencies.^{27,85} It is both rationally and intuitively appropriate to apply unconventional coagulation assays, which include both plasma and cellular blood components in the analysis, to diagnose and monitor patients with abnormal hemostasis. Whole blood contains all the intravascular factors and cells participating in physiologic and pathologic hemostasis, incorporating TF and PL-bearing cells. Whole blood assays such as thromboelastography (TEG) may provide a more truthful reflection of in vivo hemostasis than the traditionally used plasma based hemostasis assays.

Method

TEG is not a new method, but its potential use in assessing hemostatic disorders has resurfaced after it was reintroduced with new software and activators, allowing for rapid, qualitative and quantitative assessment of hemostatic function.⁵⁴ TEG is applied to whole blood, which enables a unique combined evaluation of both

the plasma and cellular components of coagulation.⁸² Originally the assay was performed on unstabilized fresh blood within 4–6 minutes of patient sampling, requiring the TEG machine to be in close vicinity to the patient. However, in a larger hospital environment with centralized laboratory facilities, an assay with such a short transfer time is rarely practical.⁸² In order to increase the time span from patient sampling to analysis, it was therefore proposed that whole blood stabilized in 3.2% citrate be used instead, with re-calcification immediately before analysis.²⁴

As with other coagulation analyses, blood samples for TEG analysis should be collected in a standardized manner and it is recommended that samples are handled without delay after receipt in the laboratory. Additionally, studies in both humans and dogs have indicated that a set time point post sampling (e.g. 30 minutes) should be used for serial measurements (Table 138.2).^{161,164}

In veterinary medicine, TEG analyses are almost always performed on citrated whole blood samples, but plasma samples can be assayed as well.¹⁶⁰ There are two different commercial machines on the market, the ROTEM produced by Pentapharm in Germany and the TEG, which is produced by Haemoscope (Fig. 138.2). Both machines function on the same principle, namely



FIGURE 138.2 The TEG 5000 in a laboratory setting.

TABLE 138.2 Results of Tissue Factor-Activated Thromboelastography Analysis on Canine Citrated Whole Blood from 18 Clinically Healthy Dogs after 30 and 120 Minutes of Storage at Room Temperature^a

	R* (min)	K* (min)	α* (deg)	MA* (mm)
30 min, mean	5.61	4.20	45.33	47.96
(range)	(2.75–8.70)	(2.30–7.65)	(27.45–58.65)	(38.95–59.00)
120 min, mean	4.91	3.34	50.90	50.19
(range)	(3.20–6.65)	(1.95–4.95)	(40.65–62.95)	(42.25–60.50)

^aR, reaction time; K, clotting time; α, angle; MA, maximum amplitude.

*Indicates that the measurement is significantly different at 30 min vs. 120 min ($p < 0.05$).

the gradual binding of a pin to the sides of a cup during clot formation. The main difference between TEG and ROTEM is that in the TEG it is the cup that oscillates and in the ROTEM it is the pin. The following is a description of the TEG method, since it is the most common.

The blood sample is loaded into a pre-warmed (37°C) oscillating sampling cup containing calcium, and the cup is raised into contact with a disposable pin. The pin is connected to a torsion wire within the instrument. The cup oscillates slowly from side to side, and as a clot starts to form, fibrin strands begin to bind the cup and pin together. As clotting is amplified and propagates, more and more fibrin is formed, which causes the amplitude of the pin's rotation to increase. The rotation of the pin is transmitted via the torsion wire to a computer which continually monitors the amplitude of the pin's oscillation. As the clot develops the computer displays a representative tracing, which begins as a straight flat line, which upon initiation of clot formation separates into two divergent lines. Data measurement can continue for up to 3 hours, and thus can include not only evaluation of the kinetics of the initiation and amplification phases of coagulation, but also assessment of strength and stability of the formed clot (Fig. 138.3).

Three types of standard TEG assays have been evaluated in veterinary medicine: native (no activator), human recombinant tissue factor activated (Innovin, Dade Behring) and kaolin activated (Haemoscope). The simplest way to perform a TEG analysis is without the use of an activator (native TEG), which offers the oppor-

tunity to evaluate the blood clotting process without the interference of any form of exogenous substance that may affect clotting. The benefit of this approach is that it is simple and inexpensive to use. The disadvantage is that there is a prolonged lag time from start of the assay and until first clot formation. Furthermore there may be an issue of increased analytical variation, although this has yet to be confirmed in veterinary medicine. In order to reduce lag time, increase sensitivity and minimize analytical variation, two different activators have been applied in veterinary medicine. Kaolin is a commercially available, "ready to use" reagent that is used to reflect the ability of blood clotting initiated via the intrinsic coagulation cascade. Tissue factor (TF), on the other hand, reflects ability of blood clotting initiated via the extrinsic coagulation cascade. The appealing aspect of TF is that it potentially reflects the physiological initiation of coagulation as it is perceived to take place in the cell based model of hemostasis. Unfortunately the TF assay is not commercially available, and therefore somewhat more laborious to perform since the reagent has to be pre-diluted.

Irrespective of which activator and reagent are used, it is well established that the reference intervals are not interchangeable at the present time; therefore each laboratory has to individually establish their own reference intervals.

Clinical Application

The ability of a laboratory assay to reveal and correlate to clinical phenotype is crucial for rational hemostasis

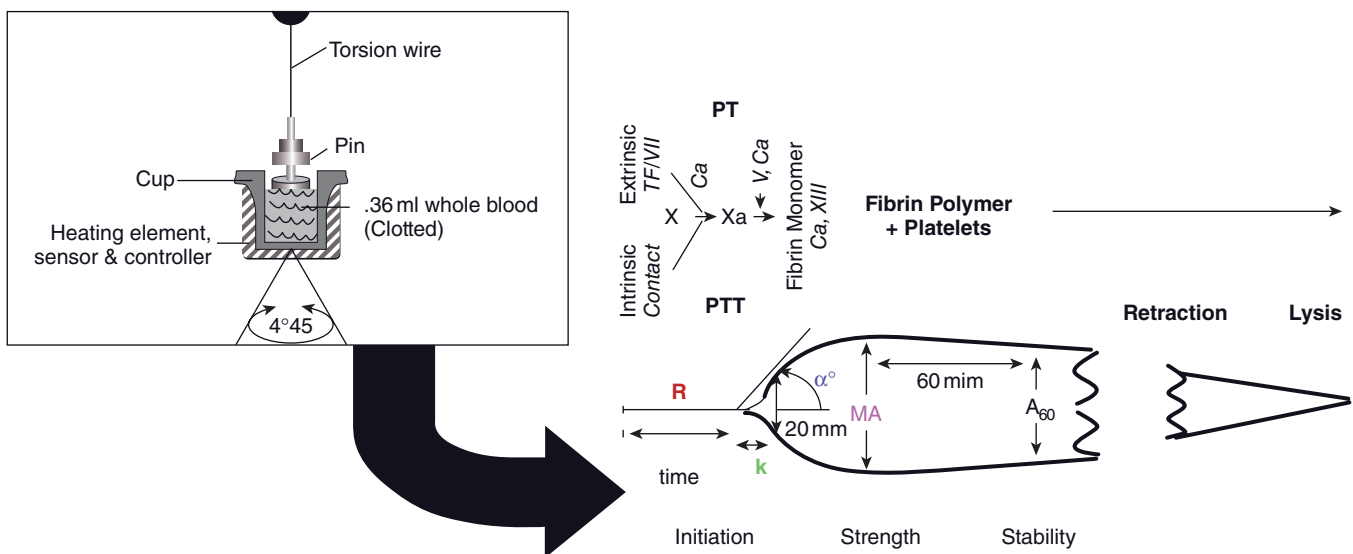


FIGURE 138.3 Example of TEG tracing with most common parameters and relationship to plasma based coagulation assays. R is the reaction time and represents the time of latency from the time that the blood was placed in the TEG analyzer until a preset fibrin formation is reached, measured as an increase in amplitude of 2mm. The clotting time (K) is the time to clot formation time measured from the end of R until an amplitude of 20mm is attained, and is a measure of the time it takes from initial clot formation until a predetermined clot strength is reached; α (or angle) represents the rapidity of fibrin build-up and cross-linking; MA, maximum amplitude; A_{60} , amplitude at 60 min; PT, prothrombin time; PTT, activated partial thromboplastin time; TF, tissue factor; Ca, calcium; VII, factor VII; X, factor X, Xa, activated factor X.¹⁶¹ (Modified from Wiinberg B, Jensen AL, Rozanski E, et al. Tissue factor activated thromboelastography correlates to clinical signs of bleeding in dogs. *Vet J* 2009;179:121–129, with permission. ©Oxford University Press.)

monitoring. The ideal hemostasis assay should therefore be able to identify both biochemical and cellular abnormalities in the hemostasis system and at the same time correlate to the clinical signs of the patient. Thromboelastography offers a welcome opportunity for point of care evaluation of overall hemostatic capability and could potentially provide clinicians with an improved ability to diagnose, monitor and predict therapeutic response in animals with bleeding and/or procoagulant and thrombotic disorders. Consequently, TEG analysis has the potential to aid in the diagnostic workup of patients with abnormal hemostasis and supplement the information received from traditional coagulation assays, such as PT, aPTT, D-dimer, and fibrinogen assays. Figure 138.4 depicts TEG tracings of the different states of hemostasis as they have been reported, mainly in humans, but also in animals.^{32,82}

Thromboelastography has been used in several veterinary studies so far, both clinical and ex vivo. The studies have mainly included pigs, dogs and cats, but also horses and alpacas have been studied and the results published.^{2,8,119,153,161} Several other species have been studied at research institutions, but the data have not been published.

A recent study in dogs demonstrated that TF activated TEG (TF-TEG) is able to correctly identify dogs with clinical signs of bleeding with both higher positive and negative predictive values than the conventional coagulation profile consisting of aPTT, PT, D-dimer, fibrinogen and platelet count, which are widely used in veterinary medicine.¹⁶²

In another study examining the application of TF-TEG as an aid in the diagnosis of DIC in dogs, it was demonstrated that TEG can be used to distinguish between different stages of DIC. Thus TEG as a test of global hemostasis may potentially provide an option for more individually tailored treatment plans for patients with DIC.¹⁵⁹

In a study where TF-TEG was used to examine the overall hemostatic state in dogs with a variety of neo-

plasias, 28 out of 49 dogs (57%) had abnormal TEG results. Overall, a total of 22 out of 49 dogs could be characterized as hypercoagulable (45%), and six were characterized as hypocoagulable (12%). The results confirm that a majority of dogs with malignancy have hemostatic dysfunction, but most importantly, it was documented for the first time that the most common abnormality is hypercoagulability.⁷¹

Thromboelastography has traditionally been used in humans to monitor hemostatic function during surgery and to optimize blood product selection and usage. The use of TEG as a transfusion guide seems to have a large potential in veterinary medicine, as it is intuitively easy to perform and interpret (Fig. 138.5). Furthermore, it has the potential to significantly optimize blood product usage and efficacy. But TEG also has the potential to test the efficacy of different types of medication in vitro.¹³²

A veterinary example of this is found in an in vitro study, where heparinase modified TEG was investigated as a possible method to evaluate the effect of therapy with the low LMWH dalteparin in dogs.⁶¹ The results show that spiking citrated canine whole blood with increasing doses of dalteparin significantly and dose dependently affects all TF-activated TEG parameters. In contrast to this, it was observed that when using kaolin as an activator there was almost no measurable dalteparin effect.⁶¹

Long-term corticosteroid therapy has been associated with an increased risk of thromboembolic complications in dogs, and TEG has been used in a small prospective pilot study in an attempt to detect the development of a hypercoagulable state in Beagles receiving oral prednisone.¹²⁶ Further studies are needed to determine the underlying mechanisms of this hypercoagulability.

Recently TEG has also been used to monitor the anti-platelet effect of various non-steroidal anti-inflammatory drugs and clopidogrel.^{14,15}

These last four studies emphasize a unique feature of the TEG assay, which suggests that it could be uti-

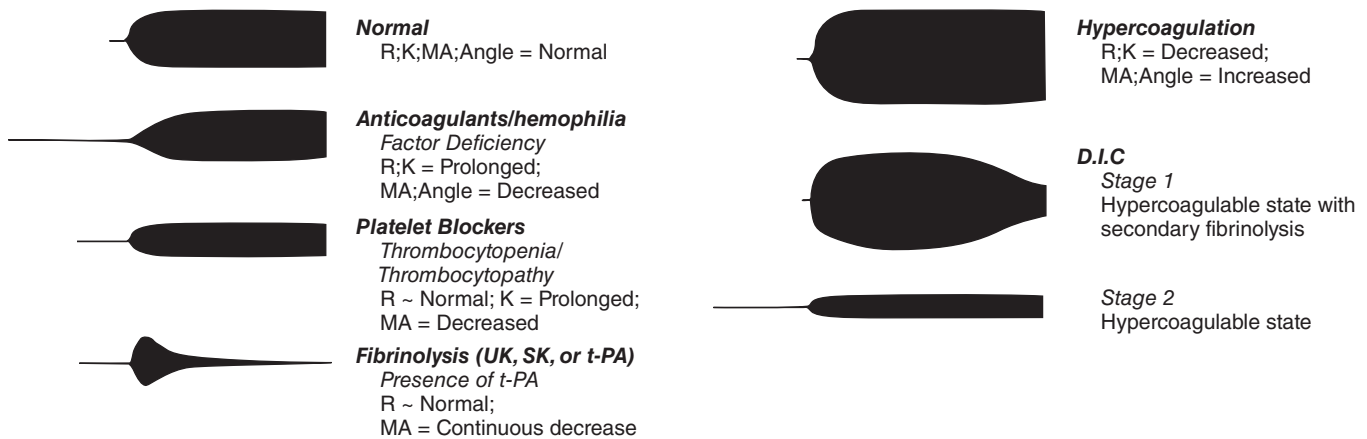


FIGURE 138.4 Pattern recognition chart of TEG tracings, compiled from human research (from Haemoscope Corporation, with permission). R, reaction time; K, clotting time; MA, maximum amplitude; t-PA, tissue plasminogen activator; DIC, disseminated intravascular coagulation.

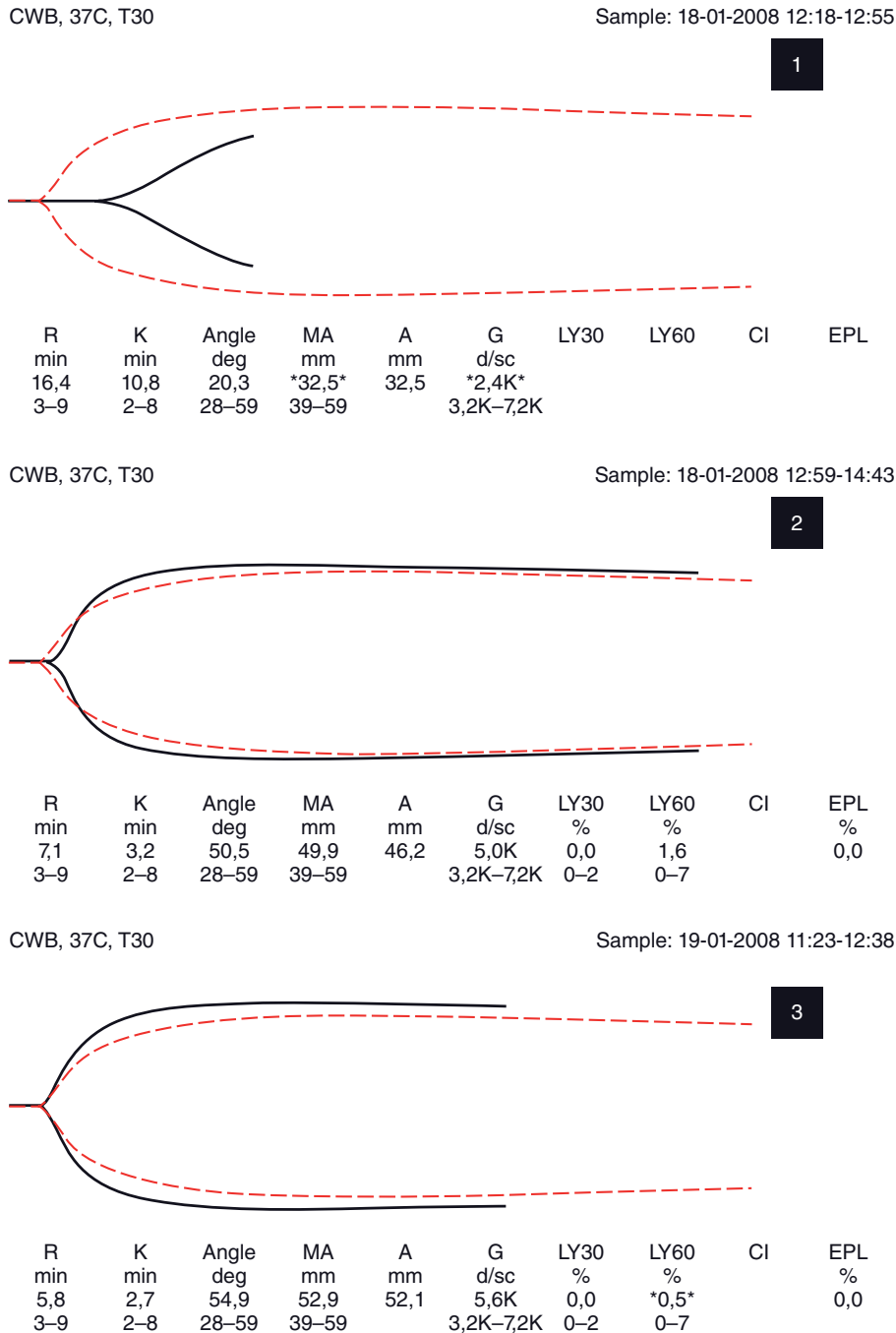


FIGURE 138.5 Clinical example, where TEG is used to guide transfusion therapy. (1) Patient sample **pre-treatment**. Prolonged R, K with decreased angle and slightly decreased MA, demonstrating coagulation factor deficiency. (2) Patient sample **pre-treatment** added fresh frozen plasma in vitro at a dosage equivalent to 10mL/kg. The results show normalization of all TEG parameters, indicating good effect of treatment. (3) Patient sample **post-transfusion** with 10mL/kg fresh frozen plasma. The results are very similar to the projected effect of treatment. R1, reaction time; K, clotting time; MA, maximum amplitude.

lized in targeting therapy of the patient towards normalization of their TEG tracings and thereby tailor dosage to meet the requirements of the individual patient.

In other species, TEG has been used in studies on cats with cardiogenic arterial thromboembolism,

and has evaluated the effects of LMWH and UFH therapy in cats with and without cardiac disease.¹ Thromboelastography has also been used to detect platelet dysfunction in a horse and to evaluate the hemostatic response in pigs undergoing pericardiectomy.^{81,118}

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Clinical Blood Typing and Crossmatching

K. JANE WARDROP

Blood Typing (see Chapter 92)

- Laboratory Testing and Submission
- Blood Typing in the Dog
- Blood Typing in the Cat
- Clinical Blood Typing Methods
- Blood Typing in Large Animals

Crossmatching (see Chapters 95 and 98)

- Indications and Limitations
- Crossmatching in Large Animals
- Standard Crossmatching Procedure
- Crossmatching using Gel Tubes

Acronyms and Abbreviations

ACD, acid citrate dextrose; EDTA, ethylenediaminetetraacetic acid; IAT, indirect antiglobulin test; NI, neonatal isoerythrolysis; RBC, red blood cell.

Blood typing and crossmatching are techniques used in the selection of blood components before transfusion. When blood typing is performed, antisera or other reagents are used to identify specific red blood cell (RBC) antigens. In crossmatching, antibodies are detected by reacting donor sera and donor RBCs with recipient sera and recipient RBCs. The primary purpose of this pretransfusion testing is to ensure that the transfused RBCs have optimal survival when transfused and do not cause harm to the recipient.

Pretransfusion testing in humans generally involves a type and screen procedure. This consists of an initial typing for ABO and Rh antigens and detection of patient antibody by mixing patient serum with commercially available reagent RBC that contain known antigens. An indirect antiglobulin test (IAT) is used during this antibody screen. When no clinically significant antibodies are detected, an immediate spin crossmatch, in which serum is mixed with saline-suspended RBCs and centrifuged immediately, can be performed to ensure ABO compatibility. If clinically significant antibodies are found, RBCs that lack the corresponding antigens are used, and an IAT crossmatch is performed. The IAT is best for detecting IgG antibodies. Use of a low-ionic-strength saline solution in the IAT further enhances its sensitivity. Techniques using microplates, solid-phase systems, and microtubes, where antiglobulin reagent is contained within a matrix of Sephadex or glass microbeads, have also been developed.⁸

Blood typing and crossmatching can also be used before transfusion in most domestic animals. The development of veterinary blood banks for animals and an increased awareness of the benefits of transfusion have resulted in increased availability and use of blood products, especially whole blood, packed RBCs, and plasma. Transfusions performed with untyped or uncrossmatched blood can sensitize recipients to future transfusions or can result in reactions that range from enhanced removal of the transfused RBCs to death of the patient. Pretransfusion testing in veterinary clinical practice is frequently less complex than that performed in the human field, largely because of a lack of appropriate reagents.

BLOOD TYPING

Laboratory Testing and Submission

Blood typing of some animal species can be performed in commercial veterinary laboratories. A partial listing of these laboratories is provided (regional veterinary diagnostic or clinical pathology laboratories may also offer these services and can be contacted).

1. Animal Blood Resources International (dogs, cats)
4983 Bird Drive
Stockbridge, MI 49285
517-851-8244

2. Equine Parentage Testing and Research Lab (horses)
102 Animal Pathology Bldg.
University of Kentucky
Lexington, KY 40546-0076
859-257-3656
3. Hematology Laboratory (horses)
Room 1012, Veterinary Medical Teaching Hospital
One Garrod Drive
University of California
Davis, CA 95616
530-752-1303

To perform blood typing, laboratories usually require the submission of either ethylenediaminetetraacetic acid (EDTA) or acid citrate dextrose (ACD) whole blood. Serum also may be required for antibody testing.

Blood Typing in the Dog

Canine RBC antigens are primarily identified by use of polyclonal antibodies generated through canine alloimmunization. DEA 1.1, an antigen capable of producing acute hemolytic reactions in sensitized animals, can be identified with a monoclonal antibody.¹ DEA typing for dogs varies, depending on the laboratory used, with some laboratories only typing for DEA 1.1, and others typing for DEA 1.1, 1.2, 3, 4, 5, and 7. Other RBC antigens may be present; however, lack of available antisera prohibits or prevents their identification. This is true for both well known antigens and newly discovered antigens. For example, DEA 6 and 8 are common antigens (DEA 6 is present in 99% of dogs; DEA 8 may be present in 40% of dogs) identified in dogs. Lack of available antisera prevents their identification by most typing laboratories. The *Dal* antigen is a newly discovered RBC antigen of dogs, and its frequency is unknown;² typing sera for this antigen also is commercially unavailable.

Blood Typing in the Cat

The best characterized blood-group system in cats is the AB system. This system consists of three blood types, A, B, and AB. Type A is the most frequent blood type, occurring in more than 95% of domestic short-hair and long-hair cats in the United States. Type-B blood occurs in less than 5% of domestic short-hair and long-hair cats; however, the frequency of type A and B in cats varies with the breed of cat and the geographic location.^{5,6} For example, as many as 45% of Exotic and British Short-hair cats and Cornish and Devon Rex cats have the B blood type. Type-AB cats are rare. Most laboratories type for both the A and B antigen in cats.

All type-B cats older than 3 months of age have high titers of anti-A antibodies. These antibodies act as strong hemolysins and hemagglutinins.⁴ Type-B cats transfused with type-A RBCs have a significant hemolytic transfusion reaction, and type-A or -AB kittens born to type-B queens are at risk of developing neonatal isoerythrolysis. Cats that have type-A blood have weak anti-B alloantibodies that produce a delayed transfu-

sion reaction characterized by premature removal of transfused cells. Type-AB cats have no alloantibodies in their plasma.

Recently, a newly recognized, non-AB blood group RBC antigen was described in domestic short-hair cats, and was called the *Mik* red cell antigen.¹⁴ The absence of this antigen was found to be associated with naturally-occurring anti-*Mik* alloantibodies, capable of eliciting a hemolytic transfusion reaction.

Clinical Blood Typing Methods

Typing of dogs and cats has been simplified for clinical use by production of commercial typing kits. Typing cards (DMS Laboratories, Inc., Flemington, NJ) contain monoclonal antibody or reagent (*Triticum vulgare* lectin for blood type-B cats) that has been lyophilized onto the card wells. A small amount of diluent and patient EDTA whole blood is added, and the card is rocked for approximately 2 minutes. Agglutination indicates a positive response (Fig. 139.1). Control wells are included with the canine typing cards to aid in reading the reac-

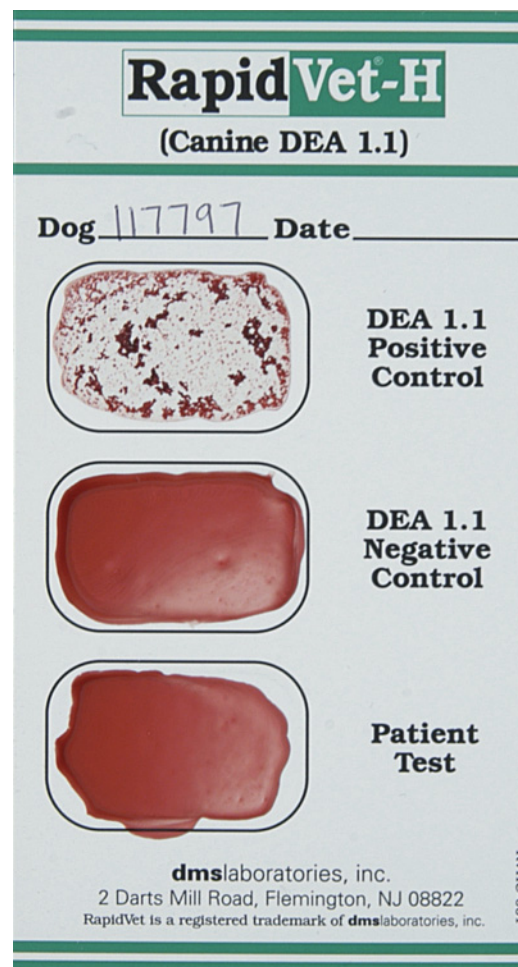


FIGURE 139.1 An example of a canine DEA 1.1 blood-typing card, with positive and negative control wells. The canine blood shows no agglutination, and is DEA 1.1 negative.

tions. For the dog, cards are available that type for DEA 1.1. Cats that have type A, B, or AB can be identified using the feline blood-typing cards. The advantage of these cards lies in their speed and simplicity. DEA 1.2 positive dogs may produce false-positive DEA 1.1 reactions on the card assay.⁷ The anti-A antibody agglutination reaction of type AB-positive cats may be weak when using the feline blood typing cards.¹¹

Typing gels are also available for the typing of DEA 1.1 in dogs and A, B, and AB in cats (DiaMed AG, Morat, Switzerland). Gel tubes contain a monoclonal antibody in a gel matrix. Antigen-antibody reactions cause agglutinates to form that then become trapped within the gel. Free RBCs pass through the gel and form a button at the bottom of the tube (Fig. 139.2). The microtubes are bound to small cards, necessitating a special centrifuge for their accommodation. Control tubes that contain gel without antibody are included to detect potential false positive reactions due to autoagglutination.

The newest test for blood group determination in dogs and cats uses a membrane dipstick technique

(Alvedia®, Villeurbanne, France) In this test, RBCs mixed with a buffer are allowed to migrate on a membrane that contains monoclonal antibodies against DEA 1.1, or against feline type A and B. In a positive reaction, RBCs will agglutinate with the antibodies and form a visible line on the membrane (Fig. 139.3).

Blood Typing in Large Animals

Blood typing for large animals is best performed by a commercial veterinary laboratory. Blood typing cards or gels are not available. Blood typing in large animals can be complex. For example, 34 different erythrocyte antigens from seven blood groups have been identified in horses.¹⁰ Cattle have at least 11 blood-group systems; sheep have seven.¹⁰ This means that identification of a blood donor with identical alloantigen and antibody profiles is difficult, if not impossible. Compatibility testing in these species, however, can be used to help avoid severe transfusion reactions by identification of existing antibodies. Alloantigens Aa and Qa are the most immunogenic of the equine RBC antigens and are responsible for the majority of equine neonatal isoerythrolysis (NI) cases.^{13,15} Mares that lack Aa and Qa are at risk of producing antibody to them upon exposure. Mares known to be at risk, either because of blood type or because of previous foaling of an affected foal, can have sera tested for antibody before foaling.

CROSSMATCHING

Indications and Limitations

Blood typing permits the identification of RBC antigens in a patient. It does not allow the detection of antibodies between a patient and a potential blood donor. In a clinical situation this is generally determined by crossmatching. Even in animals that have been blood typed, crossmatching should be performed to detect antibodies against RBCs.

Crossmatching does have its limitations. A compatible crossmatch does not necessarily indicate that the donor and recipient have the same blood type, merely that antibodies against RBCs cannot be detected. Also, the crossmatch technique may lack the appropriate sensitivity to detect some antibodies; thus it is possible to



FIGURE 139.2 An example of a canine DEA 1.1 gel test. From the left, tubes 1 and 3 are negative control tubes, tube 2 shows a negative reaction in a canine patient, tube 4 shows a positive reaction in a canine patient, and tubes 5 and 6 are unused.



FIGURE 139.3 An example of a canine DEA 1.1 membrane dipstick test. The presence of a red line by the “DEA 1.1” arrow indicates a positive reaction. The red line by the “C” arrow is a control line that indicates the test has been run successfully.

have a compatible crossmatch and still have increased removal of the transfused cells. A standard crossmatch does not detect platelet or granulocyte antibodies. It cannot predict some types of transfusion reactions, such as urticarial reactions. Nevertheless, crossmatching can be a useful technique and should be performed before transfusion. Ideally, crossmatching should be performed before all transfusions; however, where this is not possible, it should certainly be performed before those transfusions in which the recipient has a history of previous blood exposure. Crossmatching should always be performed before feline transfusions, regardless of the previous transfusion history, because of the presence of naturally-occurring antibody in the cat and the serious consequences of transfusing type-A blood into a blood type-B cat.

Crossmatching in Large Animals

Crossmatching in large animals generally requires both a saline-agglutinating technique and a technique that can detect hemolysis. Agglutination tests can be performed by most veterinary practices; however, the hemolytic tests require the addition of exogenous complement, usually provided by rabbit or guinea pig serum.¹² This serum is diluted or adsorbed first with RBCs of the species to be tested, to remove the natural heterolysins and heteroagglutinins present in the rabbit or guinea pig serum. Consequently, detection of hemo-

lytic antibodies is usually confined to the veterinary laboratory.

One crossmatching technique used in the identification of mare antibody in cases of suspected NI is the jaundiced foal agglutination test.^{10,13} In this test, colostrum from the mare is reacted with RBCs from the foal. The colostrum can also be tested with potential donor blood. Serial dilutions (1:2–1:128) with saline of colostrum are made, and one drop of EDTA foal blood is added to 1 mL of the dilution. Blood is also added to a saline control tube that lacks colostrum. The tubes are centrifuged and judged for agglutination. Positive reactions at dilutions of 1:16 or greater are considered significant in horses.

Standard Crossmatching Procedure

The crossmatching procedure described in Table 139.1 is a saline-agglutination crossmatch commonly performed by many veterinary laboratories. Some laboratories also use an IAT in this procedure, adding species-specific Coombs' reagent after the initial RBC or sera reactions have been interpreted. Antisera-treated RBCs are washed in saline, the Coombs' reagent is added in an amount and dilution determined by the manufacturer, and the mixture is incubated. The mixture is then centrifuged and the sample is checked for agglutination. A saline replacement technique is useful to detect rouleaux in species such as horses and cats, in

TABLE 139.1 Crossmatching Procedure

1. Obtain an anticoagulated (EDTA) and/or non-anticoagulated specimen of blood from both patient and donor. The EDTA tube will serve as the source of RBC (antigen), and the non-anticoagulated tube will serve as the source of serum (antibody).
2. Centrifuge and separate plasma or serum from RBC.
3. Wash RBC by adding saline or phosphate buffered saline (PBS) to a small amount of packed RBC, mixing, and centrifuging. Decant the saline and repeat 3 times, filling the tubes with saline, mixing, centrifuging, and decanting.
4. After last wash, decant supernatant and resuspend cells with saline to give a 2–4% ("weak" tomato juice) suspension of RBCs. The suspension may also be calculated; for example, 0.1 mL blood in 2.4 mL saline gives a 4% suspension.
5. Make the following mixtures by adding the indicated amount of the well-mixed RBC suspension and serum to 12 × 75 mm tubes:

Major crossmatch: 2 drops patient sera, 1 drop donor
2–4% RBC

Minor crossmatch: 2 drops donor sera, 1 drop patient
2–4% RBC

Include controls: 2 drops patient sera, 1 drop patient
2–4% RBC

2 drops donor sera, 1 drop donor
2–4% RBC

6. Incubate tubes 15–30 minutes at 37°C.
7. Centrifuge for 15 seconds (3400 rpm/1000 × g).
8. Read tubes:

Macroscopic: Examine tubes first for hemolysis. Then rotate tubes gently and observe cells coming off the red cell "button" in the bottom of the tube. Rotation of the control tubes prior to or simultaneously with rotation of the other tubes allows for optimum comparison of reactions. In a compatible reaction, i.e. where there is no antigen-antibody reaction, the cells should float off freely, with no clumping/hemagglutination (compare to the control tubes). Rouleaux formation can be falsely interpreted as a reaction. If rouleaux formation is suspected, a saline replacement technique can be used. Proceed to the microscopic examination in those tubes with weak or no obvious reactions.

Microscopic: After tubes have been viewed macroscopically, place a drop of the cells/sera mixture on a slide, apply a coverslip and examine microscopically. The RBC should normally appear as individual cells, with no clumping or rouleaux formation.

which rouleaux formation is common and can be erroneously identified as agglutination in the crossmatch procedure. Steps in the saline replacement technique³ are as follows:

1. Recentrifuge the serum or the RBC mixture when rouleaux formation is suspected.
2. Remove the serum.
3. Replace the serum with an equal volume of saline (two drops) and gently mix.
4. Centrifuge the saline or the RBC mixture at $1000 \times g$ for 15 seconds.
5. Resuspend the saline or the RBC mixture and observe for agglutination.

Rouleaux disperse when suspended in saline, whereas true agglutination remains.

Grading of reactions or antibody titration can also be performed.^{3,9}

Crossmatching Using Gel Tubes

Crossmatching using gel microtubes has been described for both the dog and the cat.^{2,14} The procedure uses saline gel microtube test cards (ID card "NaCl, enzyme test and cold agglutinins," Diamed AG, Morat, Switzerland) that contain a neutral dextran-acrylamide gel. For the major crossmatch, a 0.8% suspension of donor RBCs is prepared, using a modified low ionic strength saline solution (LISS, ID-Diluent "Vet 2," DiaMed AG, Morat, Switzerland). Patient plasma and the donor RBC suspension are added to the gel tubes, incubated for 15 minutes at 37°C, and centrifuged in a special centrifuge designed to accommodate the typing cards. The crossmatch can also be performed using gel microtubes containing anti-canine globulin (ID-gel test anti-canine globulin, Diamed AG, Morat, Switzerland). A larger tube gel crossmatching kit that uses a standard centrifuge is also commercially available (DMS Laboratories, Inc., Farmington, NJ).

Blood typing and crossmatching are valuable techniques that must be considered before transfusion in all species. Although some testing should be performed in specialized veterinary laboratories, other tests, such as saline-agglutinating crossmatching and DEA 1.1 and feline AB blood typing can be performed in most clinical veterinary practices and provide information that aids in the safe selection of blood components.

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Testing For Immune-Mediated Hematologic Disease

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Coombs' Test (see Chapters 33–35)

Methodology

Diagnostic Significance

Alternate Technologies for Antiglobulin Tests

Antineutrophil Antibody Tests (see Chapter 41)

Antiplatelet Antibody Tests (see Chapter 78)

Antinuclear Antibody Test (see Chapter 54)

Investigation of Drug-Induced Autoantibodies

Method of Antibody Detection

Drug-Induced Hematologic Disease in Veterinary Medicine

Acronyms and Abbreviations

ANAs, antinuclear antibodies; DAT, direct antiglobulin test; dsDNA, double-stranded DNA; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein-isothiocyanate; IAT, indirect antiglobulin test; ID, immunodiffusion; IIF, indirect immunofluorescence; IMHA, immune-mediated hemolytic anemia; IMN, immune-mediated neutropenia; IMT, immune-mediated thrombocytopenia; MCTD, mixed connective tissue diseases; PBS, phosphate buffered saline; PF3, platelet factor 3; PF4, platelet factor 4; PSAIg, platelet surface-associated immunoglobulins; RBC, red blood cell; RNP, ribonucleoproteins; SLE, systemic lupus erythematosus.

Immune-mediated hematologic diseases occur with some frequency in veterinary medicine. Immune-mediated hemolytic anemia (IMHA), immune-mediated thrombocytopenia (IMT), immune-mediated neutropenia (IMN), and disorders such as lupus erythematosus have all been previously described (see Chapters 33–35, 41, 54, and 78). Testing typically involves detection of antibody directed against cellular antigens. This chapter will describe specific tests available for the above named diseases.

COOMBS' TEST

The Coombs' test, also known as the antiglobulin test, is used to detect antibody and complement on the surface of red blood cells (RBCs). The test is frequently used in diagnosis of IMHA. In this disease, antibodies directed against the RBC surface cause premature destruction of the RBC: these are often incomplete antibodies that sensitize or coat RBC but fail to agglutinate them without addition of potentiators to alter the RBC zeta potential or without the addition of antiglobulin reagent. Most incomplete antibodies are IgG and are thus detected by anti-IgG serum (as contained in anti-

globulin reagent). A few IgM antibodies also can be incomplete. These incomplete antibodies produce antibody-dependent complement activation; thus antiglobulin reagents are also designed to react with complement components on the red cell surface, with the main components detected being C4 and C3. Polyspecific reagents for veterinary use typically contain anti-IgG, anti-IgM, and anti-C3. Monospecific reagents are also available.

Methodology

Two forms of the antiglobulin test are used in veterinary medicine: the direct antiglobulin test (DAT) and the indirect antiglobulin test (IAT). The DAT, that detects immunoglobulin and/or complement bound directly to RBC, is used to evaluate IMHA patients. The IAT detects the presence of unbound antibody in the serum and is not typically used in IMHA cases. An example of a tube procedure for the DAT is described in Table 140.1. In this procedure, the addition of antiglobulin reagent to the RBC with bound immunoglobulin or complement causes agglutination to occur (see Fig. 33.5).

The antiglobulin test can also be performed using microdilution plates, which allows for a greater number

TABLE 140.1 Direct Antiglobulin Test (DAT) Tube Technique

1. Place 1–2 drops of a 2–5% RBC suspension in a 10 or 12 × 75 mm test tube.
2. Wash 3–4 times with normal saline or with phosphate buffered saline (PBS). Thoroughly decant the last wash residual in order to avoid dilution or neutralization of the antiglobulin reagent.
3. Immediately add antiglobulin reagent in amounts and as diluted per manufacturer’s guidelines. Standard dilutions for canine antiglobulin are 1:2, 1:4, and 1:8. Prepare a negative control tube by adding saline instead of reagent to a tube.
4. Incubate tubes according to manufacturer’s directions.
5. Centrifuge, gently dislodge red cell button and record agglutination reactions observed as the cells come off the button. If no agglutination is apparent, a small amount of the tube contents can be placed on a slide and examined microscopically.
6. If available, add IgG-sensitized red cells (known as “check” cells) to nonreactive (unagglutinated) tests. Centrifuge and examine for agglutination.
7. If a positive DAT is obtained, monospecific reagents (if available for the species) can be used to determine whether immunoglobulin or complement (or both) are present. Freshly washed cells must be used for this step.

of dilutions of antiglobulin reagent and uses smaller volumes of reagent and RBC per well. The use of multiple dilutions has been shown to increase test sensitivity.⁴⁸

Diagnostic Significance

The antiglobulin test today is most commonly used in veterinary medicine in the diagnosis of IMHA. The use of the antiglobulin test as an aid in the diagnosis of IMHA in dogs was first described by Miller et al. in 1954, and subsequently by Lewis et al. in 1963.^{37,44} Direct antiglobulin test positive hemolytic anemia was first described in cats in 1973 by Scott et al.⁵⁴ Several studies on the use of the antiglobulin test in detecting IMHA in the horse have been described.^{38,40,43}

Sensitivity of the antiglobulin test in detection of IMHA is moderately low, with sensitivity as low as 48% reported in dogs.²⁶ False negative test results may be secondary to factors such as incomplete washing of RBCs, insufficient RBC-bound antibody, elution of Ig or C3, or to a prozone effect.^{48,63} Interestingly, positive results in the absence of IMHA can also occur. One study described a high incidence of C3b bound to RBC in dogs with infections, inflammatory disorders, and myeloproliferative and lymphoproliferative diseases.⁵⁶ Hemolytic disease in these dogs was minimal or absent. The positive DAT was felt to be useful in dogs with signs of hemolytic disease, but needed to be interpreted with caution in other conditions. In another study using cats, 16 of 20 anemic cats were DAT positive. Eleven of the 16 were FeLV positive, and the remaining five positive cats had inflammatory

disease or hemoplasmosis. Conservative interpretation of a positive test in the absence of hemolysis was recommended.¹³

Alternate Technologies for Antiglobulin Tests

Alternate test modalities have been developed to enhance test sensitivity. Most of the alternate technology associated with antiglobulin testing has been designed to either increase the sensitivity of the test or to lessen the subjectivity associated with assessment of agglutination. Enzyme-linked antiglobulin tests appear to enhance sensitivity, but are difficult to perform and also decrease the specificity for IMHA.^{3,27} Flow cytometry has been shown to be a sensitive diagnostic technique for IMHA in dogs and horses, with some decrease in specificity.^{51,67} However, the test necessitates the availability of a flow cytometer in the laboratory. Antiglobulin gel tests incorporate antiglobulin into a gel matrix. An RBC suspension is dispensed into the reaction chamber of a microtube containing the gel, and tubes are incubated and centrifuged. The microtubes may be held together in a small plastic card, which necessitates a specific centrifuge. Red blood cell agglutinates become trapped and remain stable in the gel, while free RBCs pass through and form a button at the bottom of the tube. The end-point reactions are more stable than conventional tube agglutination reactions. Strong positive reactions remain at the top of the gel, while RBCs involved in negative reactions pellet to the bottom. A gel antiglobulin test is available for dogs (DiaMed, Switzerland); however, comparison studies between this canine gel test and other types of antiglobulin testing are lacking.

ANTINEUTROPHIL ANTIBODY TESTS

IMN has been described in dogs, cats, and horses.^{7,12} The disease is frequently diagnosed by exclusion of other diseases known to cause neutropenia, supportive bone marrow findings, and by response to corticosteroids. Direct diagnosis of IMN involves detection of antineutrophil antibody; however, antineutrophil antibody tests are not widely available. Previous techniques for detection have included leukoagglutination, phagocytic inhibition, indirect immunofluorescence, and flow cytometry.⁶⁴ In one study, an indirect leukoagglutination test was used to determine antineutrophil antibodies in two giant Schnauzers.⁶² The sera of these neutropenic dogs were incubated for 30 minutes at 37°C with saline washed buffy coat cells harvested from the patient’s blood. The cells were rewashed, incubated with antiserum to canine IgG, IgM, and C3, resuspended, and applied to glass slides. The slides were stained and viewed with a microscope. Serum from the patients but not from a control dog demonstrated agglutination of the patient’s neutrophils, and a tentative diagnosis of IMN was made. A leukoagglutination method has been described in the cat, but the technique used was deemed difficult and fraught with interpreta-

tive problems.¹⁰ An indirect immunofluorescence test, using paraformaldehyde-fixed cat neutrophils incubated with patient serum and tagged with fluorescein isothiocyanate (FITC) conjugated antiglobulin was described for cats, but failed to identify any antineutrophil antibody in 55 feline neutropenic patients.¹⁰ A phagocytic inhibition test has been described in the horse as a method for detection of IMN.²⁵ In that test, the presence of neutrophil antibody in patient serum was indicated by inhibition of normal neutrophil phagocytic activity, but the assay was felt to be relatively insensitive.

Both direct and indirect flow cytometry are described as being the most sensitive tests for antineutrophil antibodies. A flow cytometric method using unfixed equine neutrophils treated with rabbit antineutrophil serum and fluorescein-conjugated goat anti-rabbit IgG antibodies has been described.²⁴ In the dog, patient serum was incubated with paraformaldehyde-fixed neutrophils and subsequently incubated with fluorescein conjugated rabbit anti-dog IgG.⁶⁵ Factors such as alloantibodies, immune complexes, or neutrophil donor variation did not appear to be present. Use of this test in 12 dogs with neutropenia demonstrated antibody in five out of six dogs with a clinical diagnosis of IMN and lack of antibody detection in six out of six dogs with neutropenia associated with other diseases.⁶⁴

ANTIPLATELET ANTIBODY TESTS

IMT has been described in dogs, horses, and rarely in cats, and the pathologic mechanisms are described in more depth in Chapter 78.^{28,33,41} The disease may be primary (idiopathic), or secondary to a variety of underlying conditions caused by infectious agents (primarily tick borne), neoplasia, or associated with drug administration.³³ In dogs with primary IMT, the platelet reactive antibodies are frequently reported to be of an IgG class and have been identified to be directed against the platelet surface glycoprotein gpIIb/IIIa (CD41/CD61), the fibrinogen receptor antigen.^{35,55} As a consequence, the antibody-coated platelets are rapidly cleared by macrophages in spleen or other tissues.⁶⁹ In secondary IMT, platelet destruction can be consequent to binding of immune-complexes to platelet Fc receptors, exposure of new antigens after membrane damage, nonspecific adsorption of foreign antigens (i.e. drugs), or newly generated antigens produced during the course of infectious or neoplastic diseases.^{33,53,69} Several techniques have been developed to detect platelet surface-associated immunoglobulins (PSAIg) in primary IMT.⁵³ Initial methods to identify antibodies bound to platelets of human patients with IMT measured the total IgG concentration in platelet lysates.^{11,34} These methods are not considered appropriate anymore, because subsequent studies identified the capacity of platelets and megakaryocytes to internalize plasma proteins into alpha granules including large amounts of IgG.^{11,34}

Early assays to detect platelet antibodies in dogs mainly consisted of the platelet factor 3 (PF3) test and a direct immunofluorescent test to identify antibodies on megakaryocytes in bone marrow. The PF3 test was an example of a functional assay in which binding of platelet antibodies induces the release of PF3 and alters the clotting time.^{23,61} The PF3 test lacks diagnostic specificity and is no longer used.^{23,33} The direct immunofluorescent test for megakaryocytes is infrequently requested because it requires a bone marrow aspirate and has variable sensitivity.³³

Indirect methods to detect serum antibodies that have binding specificities for platelets (platelet bindable antibodies) and direct methods to detect PSAIg have been described using enzyme-linked immunosorbent, immunoradiometric, and immunofluorescent (microscopy or flow cytometry) labeling techniques.^{30,34,42,55,69} Indirect assays to detect antiplatelet antibodies in canine serum are infrequently used, because levels of circulating platelet bindable antibodies are typically low; therefore diagnostic sensitivity of the test is low (average sensitivity reported to be 60%).^{29,30,36,42} Radioimmunoprecipitation methods described in dogs with primary IMT have identified specific autoreactive antibodies, but are laborious and not routinely available.^{35,55}

Direct assays using flow cytometry to detect PSAIg are the most common methods currently in use for dog, horse, and cat blood samples.^{28,33,41,69} At the Immunology Laboratory at Kansas State University College of Veterinary Medicine, platelet-rich plasma is prepared from EDTA anticoagulated samples and unbound immunoglobulins are removed by three washes in Tyrode's buffer. A species-specific FITC-conjugated monoclonal antibody against canine or feline IgG (polyclonal antibodies specific for equine IgG, IgM, and IgA are used for horses) is incubated with an aliquot of washed platelet suspension. Ten thousand platelets are counted and their signals recorded by the flow cytometer. The percentage of platelets coated with Ig is defined by the proportion of counted platelets that produces fluorescent signals above the background. The background represents the nonspecific binding of proteins to the platelet surface and is determined by the reaction of a FITC-conjugated isotype control antibody (Fig. 140.1).⁶⁹ Direct immunofluorescent assays for IMT have a high diagnostic sensitivity (90–95%) and a low diagnostic specificity.³³

It is important to mention that these direct labeling techniques are used to identify the presence of surface-associated immunoglobulins on platelets and do not identify autoantibodies specific to platelet glycoproteins. Therefore, the sensitivity and specificity of any method for the detection of PSAIg should refer to its ability to detect immunoglobulins on the platelet surface, rather than correlating with the subjective clinical probability of primary IMT.^{11,22,53} The diagnosis of primary IMT, in both human and veterinary medicine, is still based on the clinical impression, the exclusion of known causes of thrombocytopenia, and response to treatment.^{22,55,61} The detection of PSAIg, hence, is not

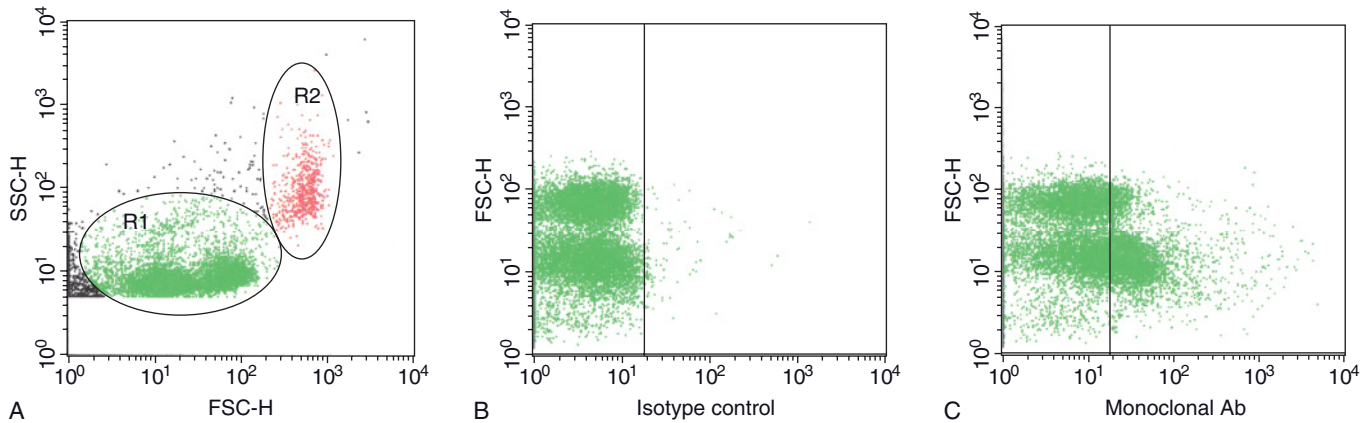


FIGURE 140.1 Dot plot results of the flow cytometric direct immunofluorescent test to detect PSAIgG on canine platelets. Each dot represents platelets/cells in a concentrated preparation of platelet-rich plasma (PRP). (A) Scatter plot of PRP demonstrates that the size and complexity of platelets (R1) is less than that of contaminating RBCs (R2) (SSC-H, side scatter height or complexity; FSC-H, forward scatter height or size). Two fairly distinct populations of platelets of different sizes are noted. (B) FSC-H versus fluorescence plots gated on platelets (R1) incubated with an isotype control antibody (FITC-labeled mouse IgG) which demonstrates background fluorescence to the right of the vertical line (1% of the gated platelet population); (C) FSC-H versus fluorescence plots gated on platelets (R1) incubated with a monoclonal antibody specific to canine IgG; platelets shifted to the right of the vertical line (42% of the gated platelet population) demonstrate binding of the fluorescent monoclonal antibody to platelet-bound IgG (PSAIgG).

pathognomonic of primary IMT and true-positive results are also found in other disorders with concurrent immune-mediated platelet destruction (secondary IMT). For this reason, the American Society of Hematology first defined the detection of PSAIg inappropriate and unnecessary in primary IMT patients.^{16,22} However, more recent literature suggests that PSAIg assay could be useful as a screening test for primary IMT due to its high sensitivity.^{22,61} A negative result, in fact, rules out an immune-mediated mechanism as the basis of the platelet destruction, whereas positive results must be confirmed by more specific assays, such as the monoclonal antibody-specific immunomobilization of platelet antigen assay (MAIPA), which is available in human medicine.^{6,22,33,36}

A major disadvantage to the PSAIg assays is the poor stability of the sample that can lead to false positive results.^{29,68,69} In fact, platelets can be activated by an improper blood drawing, poor sample handling (i.e. unnecessary agitation of the tube), low storage temperature, prolonged length of time between blood draw and sample preparation (storage time) and centrifugation steps, with subsequent exposure on the platelet surface of IgG contained within the alpha granules.^{11,31,34,47,68-70} In the flow cytometric PSAIg test, platelet shape changes can be evaluated as an indicator of activation. When activated, the platelet population (forward-scatter versus side-scatter plots) spreads from the normal well-defined oval shape to an elongated and narrower scatter plot (Fig. 140.2).⁴⁷

Flow cytometry methods using whole blood samples have been proposed as alternative methods to minimize in vitro platelet activation that occurs during the preparation of platelet-rich plasma.⁵⁹ However, a prolonged contact with leukocytes before platelet separation can trigger platelet activation.^{31,59} Because the principal

sources of platelet activation occur in a pre-analytical phase and are neither predictable nor trackable (i.e. shipment condition), positive results must be interpreted with caution and in the context of clinical and supporting laboratory findings.

ANTINUCLEAR ANTIBODY TEST

Antinuclear antibodies (ANAs) are a heterogeneous population of antibodies reacting against self nuclear components.^{32,66} The identification of ANA specificities in humans is of diagnostic and prognostic value since particular autoimmune disorders are associated with the presence of specific autoantibodies.³² The indirect immunofluorescence (IIF) assay is the most frequently used screening test for detection of circulating ANAs in both human and veterinary medicine.^{21,32} Briefly, serial dilutions of serum from patients are applied to nuclear-rich tissue substrates fixed on glass slides, either rat liver sections or human monolayer epithelial cells (e.g. Hep-2, HeLa, Vero).^{18,21} A secondary anti-species-specific antibody, typically conjugated with FITC, is applied to the sections, and the slide is then washed, after incubation, to remove unbound antibody. A specific ANA reaction is identified if the fluorescent staining is specific to the tissue substrate nuclei using fluorescence microscopy (Fig. 140.3).^{21,32,50}

In humans beings, the pattern of nuclear fluorescence suggests the type of antibodies present and guides in selecting additional tests for the detection of antigen specificity and disease association. A homogeneous or peripheral nuclear staining usually reflects the presence of antibodies directed against chromatin constituents, such as double-stranded DNA (dsDNA) or histones. In people this is characteristic of systemic lupus erythema-

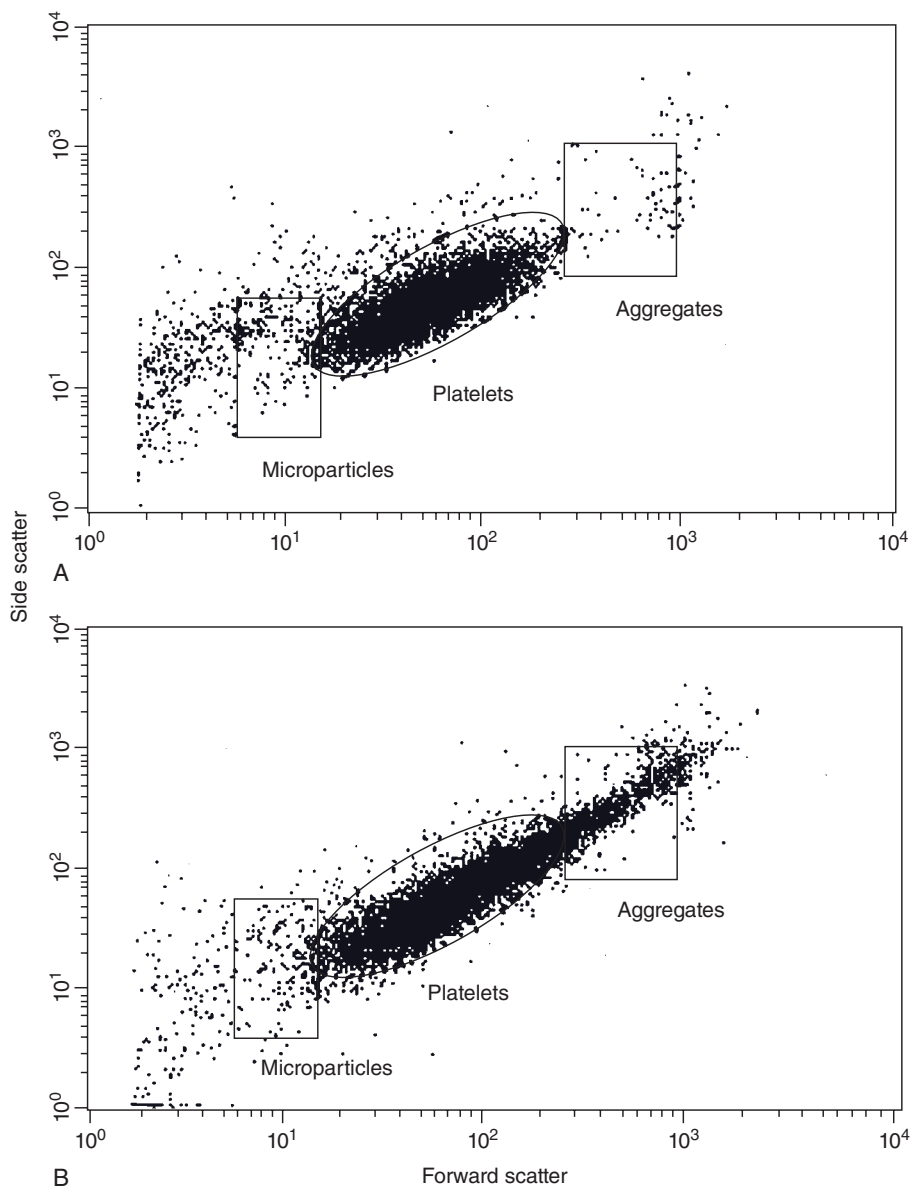


FIGURE 140.2 Forward-angle versus side-angle light scatter plots. (A) The scatter of non-activated platelets is a characteristic well-defined oval shape. (B) Platelets activated with 20 ng/mL phorbol myristate acetate. The platelet population spreads to form an elongated and narrower scatter plot. Platelet fragments (microparticles) and platelet aggregates are produced upon activation and are discernible as two distinct populations with a lower and higher forward scatter, respectively. (Reproduced from Moritz A, Walcheck BK, Weiss DJ. Flow cytometric detection of activated platelets in the dog. *Vet Clin Pathol* 2003;32:6–12, with permission.)

tosus (SLE) or drug-induced lupus. The speckled staining is the most frequently identified pattern but is not specific for a particular autoimmune disease. In human beings it is seen in the presence of antibodies to non-DNA components such as ribonucleoproteins (U1-RNP), Scl-70, Smith antigen (Sm), and Sjögren's syndrome sicca antigens A and B (SS-A, SS-B), that are associated with mixed connective tissue diseases (MCTD) and SLE, scleroderma, SLE, and Sjögren's syndrome sicca complex, respectively. The centromere and nucleolar stainings are more often associated with scleroderma, polymyositis, and other connective tissue diseases. In the majority of the cases of IIF positivity, serum samples are further analyzed for the determination of

the specificity of the auto-reactive antibodies.³² The most common secondary tests used include enzyme-linked immunosorbent assay (ELISA), immunodiffusion (ID), radioimmunoassay and immunoblot.^{32,66} The double immunodiffusion technique of Ouchterlony has been used in dogs with positive immunofluorescence for ANAs.¹⁹ The serum tested diffuses toward lyophilized mammalian nuclear antigens and forms a visible line where the immune complexes precipitate. Specific human sera reactive to predetermined extractable nuclear antigens are used to establish the specificity of the precipitating canine sera.¹⁹

The application of ANAs in veterinary medicine is still restricted to the diagnosis of SLE.²¹ The lupus ery-

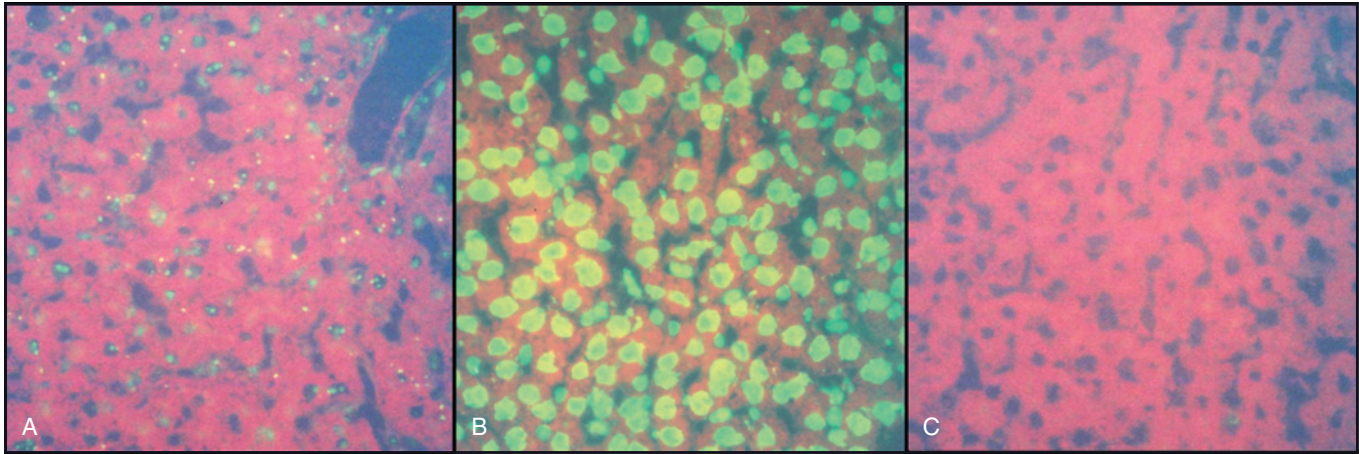


FIGURE 140.3 Antinuclear antibody patterns of canine serum reacting in the immunofluorescence test using rat liver substrate. Three separate canine serum samples reacted with FITC-conjugated anti-canine IgG to produce (A) a nucleolar staining pattern of hepatic cells; (B) a diffuse nuclear staining of hepatic cells; and (C) no specific staining of nuclear material. (Reproduced from Paul S, Wilkerson MJ, Shuman W, et al. Development and evaluation of a flow cytometry microsphere assay to detect anti-histone antibody in dogs. *Vet Immunol Immunopathol* 2005;107:315–325, with permission.)

thematous cell test (LE test) also has been used to diagnose this disorder, but the test has a high incidence of false negative and false positive results, and is uncommonly used (see Chapter 54). SLE is rarely reported in cats and horses, and lack of comparative data makes difficult the interpretation of ANA titers.^{15,18,60} Antinuclear antibody titers in cats diagnosed with SLE are usually low (below 1:40) and overlap with the titers of healthy subjects. Furthermore, titers as high as 1:100 can transiently be found in both FeLV and FIP positive cats.^{18,60}

In dogs, a positive ANA titer is expected in 97–100% of cases of SLE, but the sensitivity of immunofluorescence varies between laboratories and substrate employed.^{8,18} Antinuclear antibodies can also be produced in many inflammatory and infectious diseases, and a positive immunofluorescence result must be interpreted in the context of clinical signs and laboratory data.^{21,58} Therefore a positive ANA test is listed in the revised American Rheumatism Association diagnostic criteria for people adapted for dogs, but should not be considered the sole criterion for the diagnosis of SLE.^{9,21}

Positive ANAs have been described in dogs with non-autoimmune disorders such as in atopic dermatitis, onychodystrophy, and black hair follicular dysplasia, and in subjects sero-positive for *Bartonella vinsonii* subsp. *berkhoffii*, *Ehrlichia canis*, or *Leishmania infantum*.^{17,18,49,58} Dogs with non-primary immune-mediated disorders tend to have low positive titers (91% \leq 1:256), but positive titers up to 1:4000 occasionally can occur in dogs.^{8,17,21,46,58} In contrast, higher immunofluorescence titers were found to be clearly correlated with signs of autoimmune diseases (1:100 on Hep-2)²¹ and titers \geq 1:640 were considered to be a good predictor of immune-mediated diseases in dogs.⁵⁷ Positive titers can also be found in healthy dogs, especially when cryopreserved sections of rodent liver are employed.^{18,21} The

appropriate threshold titer of positivity must be determined in each laboratory based on a control population and the type of substrate.²⁰ In general, high titer cut-offs (e.g. 1:100) are required with rat liver substrates to decrease the number of false positive results.²¹ Hansson et al. found the Hep-2 substrate to be superior to rat liver sections not only for its high specificity (no false positives at 1:25 dilution in 100 healthy dogs), but also because it provides an easier and more consistent pattern discernment.²¹ Using Hep-2 slides, two major patterns are recognizable in dogs: the homogeneous, characterized by an intense fluorescence staining of the chromosomal area in mitotic cells; and the more commonly occurring speckled pattern, chromosome-negative.²¹ An association between homogeneous immunofluorescence pattern and SLE has been reported to occur in dogs, analogous to humans.¹⁹ However, Hansson et al. failed to demonstrate an association between speckled immunofluorescence patterns and specific canine autoimmune diseases.¹⁹ Likewise, a correlation between immunofluorescence patterns and ANA specificities was not found when the sera were analyzed with Ouchterlony ID, immunoblot, and ELISA, even if reacting bands were exclusively found in speckled patterns.⁶⁶ These results are possibly due to the lack of well-defined diagnostic criteria for the different autoimmune diseases in dogs and their overlapping clinical signs.^{19,20} Furthermore, several canine auto-antigens differ from human antigens and are absent in the commercially available extracts made for the examination of human sera.^{18,19,66}

In contrast to humans beings, the majority of ANAs in dogs are not directed against nucleic acid in native dsDNA but against nuclear proteins such as histones and RNP.^{19,45} Autoantibodies against several individual histones (H1, H2A, H3 and H4) are increased in canine SLE whereas antibodies to histone-DNA complexes are rare.^{8,45,66,50} Some anti-RNP autoantibodies are found in

both human and canine ANA-positive samples, such as antibodies directed against the proteins U1-70K, RNP A, RNP C, Sm, and SS-A.^{8,45} Other autoantibodies, such as the so-called anti-type 1 (anti hnRNP G) and anti-type 2, are confined to dogs and many are yet to be identified.^{19,45,66}

INVESTIGATION OF DRUG-INDUCED AUTOANTIBODIES

Drugs can induce the formation of antibodies, either against the drug itself, against intrinsic cellular antigens modified by the drug, or against both (see Chapters 15 and 16). This has been most frequently described with drug-induced immune hemolytic anemia in humans.^{2,14} Drug-dependent antibodies require that the drug be present. Drug-independent antibodies react similarly to true autoantibodies (i.e. no drug is needed to demonstrate the presence of antibody). Drugs can bind loosely or firmly to cell membranes and antibodies may be made to (1) the drug itself, necessitating drug binding for in vitro detection; (2) cell membrane components; or (3) part drug, part membrane components, producing an in vitro reaction typical of an immune complex mechanism.

Method of Antibody Detection

Drug-induced anti-RBC antibodies in human are usually associated with a positive DAT. For further investigation of suspected hemolysis due to drug-dependent antibodies, special methods are used. As an example, for detection of antibodies to penicillin, drug-treated RBCs are prepared. Washed, packed group O RBCs are incubated for 1 hour with penicillin dissolved in a barbital buffer. The cells are then washed and stored in PBS. An aliquot of untreated RBCs in the same buffer is also prepared. The patient serum is mixed with a 5% saline suspension of the drug-coated cells and incubated at 37°C for 1 hour. The sample is then centrifuged, and examined for hemolysis or agglutination. The cells are also tested by an indirect antiglobulin technique.⁵ For demonstration of immune-complex formation involving drugs, the patient serum is incubated with a suspension of the drug in question. The mixture is then added to enzyme-treated group O RBCs, incubated, and centrifuged and examined as described for the drug-coated cells. A similar test for antibodies directed against drug metabolites has been described.⁵ Drug-independent antibodies react similarly to most IMHA cases, with a positive DAT test result.

Diagnosis of drug-induced immune granulocytopenia or thrombocytopenia is generally considered when there is a temporal association of the drug and the cytopenia, rather than by actual detection of antibody in the presence of drug. An exception would be heparin-induced thrombocytopenia, a condition described in human patients receiving unfractionated or low molecular weight heparin. Immunogenic complexes formed by heparin and platelet factor 4 cause antibody forma-

tion, and these anti-heparin/PF4 antibodies can be detected with commercially available immunoassays.¹

Drug-Induced Hemolytic Disease in Veterinary Medicine

Drug-induced immune hematologic disease has been poorly documented in veterinary medicine, with most reports showing a temporal association of a drug with hematologic disease, but no actual testing for drug associated antibody. Penicillin induced hemolytic anemia has been described in horses.^{4,39,52} The Coombs' test was positive in these horses, and their sera produced agglutination of penicillin-coated RBCs. In one horse, anti-penicillin antibody titers decreased progressively throughout the period of observation.⁵²

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Evaluation of Neutrophil Function

STEFANO COMAZZI

Specimen, Storage and Neutrophil Isolation

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Acronyms and Abbreviations

BAL, bronchoalveolar lavage; C5a, complement component C5; CAT, catalase; CV, coefficient of variation; DCF, 2,7-dichlorofluorescein; DHE, dihydroethidium; DNA, deoxyribonucleic acid, EDTA, ethylenediaminetetraacetic acid; F-actin, filamentous polymeric form of actin; FMLP, N-formyl-methionyl-leucyl-phenylalanine; G-actin, globular monomeric form of actin; H2-DCFDA, dichlorodihydrofluorescein diacetate; LTB₄, leukotriene B₄; MCLA, methyl cypridina luciferin analog; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; NBT, nitroblue tetrazolium salt; PAF, platelet activating factor; PMA, phorbol myristate acetate; RBC, red blood cell; ROS, reactive oxygen species; SOD, superoxide dismutase; TdT, terminal deoxyribonucleotidyl transferase; TNF- α , tumor necrosis factor alpha; TUNEL, terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labelling; ZAS, zymosan activated serum.

Neutrophils play an important role in nonspecific immunity as the first defense against microbes, mainly of bacterial and fungal origin. Deficits in neutrophil function lead to increased susceptibility to infectious diseases, and have been reported in both congenital and acquired diseases (see Chapter 42).

Nonspecific immunity *in vivo* is related not only to neutrophil function but also to several conditions, including vasopermeabilization, priming and opsonization, cytokines, complement factors, soluble mediator secretion, and interaction between neutrophils and extracellular matrix or other inflammatory and endothelial cells. Testing neutrophil function *in vivo* is quite complex and does not permit discrimination between direct and indirect alterations; thus *in vitro* tests, that are more standardized, are more useful in most pathological conditions and allow testing the direct effect of different substances, drugs, and activators on neutrophils.

SPECIMEN, STORAGE AND NEUTROPHIL ISOLATION

Theoretically, a purified population of neutrophils supplies more definitive informative information about functional abnormalities. However, isolation techniques are reported to activate cells;⁴ thus, sometimes, it is preferable to use techniques requiring whole blood (such as flow cytometry or chemiluminescence) in order to limit manipulation and activation of cells. Neutrophils from milk, exudates, and bronchoalveolar lavage (BAL) fluid can also be tested. Peripheral blood or fluids must be collected in anticoagulated tubes. Definitive studies on the influence of different anticoagulants on neutrophil function in animals are not available but heparin is generally preferred, especially when *in vitro* activation of neutrophils is desired. However, EDTA as well as acid-citrate-dextrose and citrate are often used as good alternative choices in many studies in dog, pig

and ruminants. Analysis can be delayed for up to 24 hours after collection, even though the samples should ideally be processed within 4 hours. Samples should be stored at room temperature. In order to avoid neutrophil activation, plastic instead of glass tubes should be used and sterility of all solutions should be carefully maintained.

Neutrophil Isolation by Lysis

The simplest methods of preparation of blood leukocytes is osmotic lysis of red blood cells (RBCs), adding distilled water to an aliquot of whole blood followed by restoring isotonicity with a hypertonic solution. Red blood cells are mechanically ruptured while leukocytes are more resistant, and RBC membrane debris is removed by washing steps. This technique is very simple but it does not allow isolation of leukocyte subpopulations and the percentage of purity of neutrophils is similar to that of whole blood. On the other hand, the method induces only minimal activation of neutrophils. A modification of this technique is the method described by Carlson and Kaneko¹ and has been widely used in ruminants. It involves osmotic lysis after removal of the buffy coat that containing most of the mononuclear cells. The method yields a high percentage of granulocytes, but eosinophils cannot be separated from the neutrophils. The method does not provide good purity and recovery in non-ruminant species.

Red blood cell lysis can also be performed by means of chemical solutions such as tris-buffered ammonium chloride. Unfortunately, this technique frequently interferes with some neutrophil functions or induces apoptosis; therefore it should be limited to flow cytometric evaluation of membrane antigens.

Sedimentation Techniques

Neutrophils can be enriched by adding an equal volume of dextran solution to whole blood. Dextran is a polysaccharide solution that induces rouleaux formation by increasing sedimentation of RBCs and mononuclear cells. After 30 minutes the granulocyte-rich supernatant is collected, centrifuged, and washed. This method has been described in humans and dogs,^{13,34} as a single step or followed by gradient centrifugation and it is particularly useful in small animals because it requires a minimal amount of blood. However, isolated cells are characterized by low purity and dextran can partially influence neutrophil function.

Gradient Centrifugation Techniques

Several isolation techniques use colloidal suspensions at different densities to concentrate cells according to their specific gravity. Continuous gradients have been used, but discontinuous gradients are more feasible and adaptable to routine isolation of cell populations. The most frequently used technique in veterinary medicine is separation using a gradient of discontinuous Percoll®

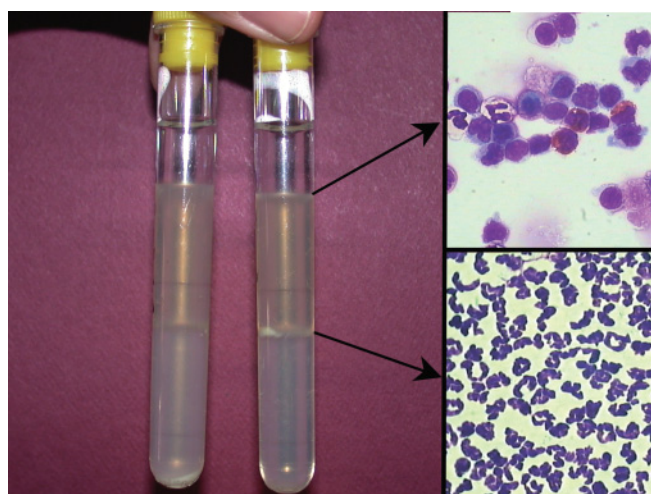


FIGURE 141.1 Isolation of blood neutrophils using a discontinuous Percoll gradient. After centrifugation a neutrophil-rich band is located at the interface between the two Percoll densities. (Courtesy of V. Spagnolo.)

silica particles coated with polyvinyl pyrrolidone. For canine neutrophils, two different densities (1.092 and 1.071 obtained by using 74% and 55% of Percoll respectively) are layered and leukocyte suspensions are put on the top of the tube.³⁷ After centrifugation, a neutrophil-rich band is easily recognizable at the interface between two different densities (Fig. 141.1). This layer can be removed and after washing provides a very pure population of neutrophils. The method can be adapted to different species by adjusting the densities of Percoll: in swine 65% and 55%; in bovine 70% and 50%; and in the horse 75% and 60% concentrations of Percoll can be used. The main advantage of this technique is the high purity and recovery of neutrophils, especially in healthy animals. However, cell densities frequently vary with physiologic and disease conditions, which could lead to failure to isolate atypical or activated cells.²⁶

A similar technique uses another commercially available polymer, Ficoll-Paque®, which is able to separate mononuclear cells and platelets (at the top) from granulocytes and RBCs (in the bottom of the tube) after centrifugation. This method is widely used in human medicine but it does not provide advantages in veterinary medicine in comparison with Percoll gradients.

Finally, an interesting method using a discontinuous metrizamide gradient has been developed and reported to isolate bovine neutrophils with high purity and yield.⁶ This technique is reported to minimally interfere with neutrophil function but it has not been widely used.

Magnetic Beads Separation

Recently, separation methods using magnetic beads have been developed in cattle.²⁵ Briefly, magnetic beads are labelled with anti-bovine neutrophil monoclonal antibodies and then incubated with leukocyte suspen-

sions. Leukocytes bound to the beads are then captured using the magnetic system while unbound cells (mononuclear and other granulocytes) are eluted and discarded. After removal of the magnetic field, collected neutrophils show a high percentage purity and yield that makes the method useful. However, this technique requires neutrophil-specific antibodies that do not recognize other polymorphonuclear cells. Moreover, incubation with some antibodies could induce activation of neutrophils, thus influencing function tests. For these reasons, antibodies should be chosen carefully.

CLASSICAL TECHNIQUES FOR EVALUATION OF THE PHAGOCYTOTIC PROCESS

Many different techniques are described for evaluation of phagocytosis, some of which are able to evaluate the whole phagocytic process while others are used to discriminate single steps of phagocytosis (i.e. rolling, adherence, random migration and chemotaxis, particle uptake and killing). Tests for a specific step of phagocytosis are useful because many diseases or conditions lead to alterations in a single function without perturbing the whole phagocytic process. On the contrary, tests for the whole phagocytic process are easier to perform and give a more complete picture of complex interactions with nonspecific immunity. Thus a panel of tests for simultaneous evaluation of different steps of phagocytosis is strongly recommended in order to correctly assess neutrophil function.

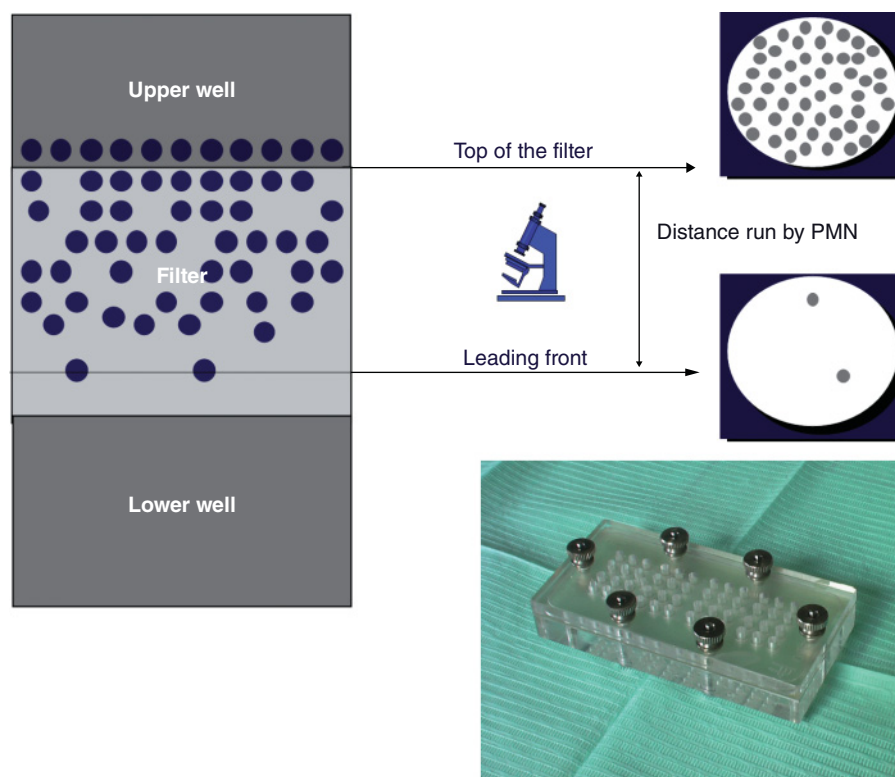
Cellular Movement and Chemotaxis

The simplest technique to evaluate cellular movement is microscopic detection of conformational changes of neutrophils after activation. Activated neutrophils show a bipolar configuration within a few minutes after exposure to activating agents with a gradual return to a spherical shape.²⁹ However, this method is difficult to standardize and does not distinguish spontaneous motility, random migration (chemokinesis), and directional migration (i.e. chemotaxis). Many chemoattractant substances have been recognized but some of them (interleukin-8, zymosan-activated serum [ZAS], casein, LTB₄, C5a, TNF- α , PAF and formylated peptides such as FMLP) are more commonly used in chemotactic tests. However, some species differences have been recognized. Neutrophils from cows, pigs, and cats do not respond to formylated peptides and those from dogs require a priming agent in order to express enough receptor for such substances.¹¹

A frequently used method to test chemokinesis and chemotaxis is the migration assay using a modified Boyden chamber.³⁶ Briefly, suspensions of isolated neutrophils are put into the upper wells of a migration chamber while chemoattractant is added in the lower well. For evaluating random migration the addition of chemoattractant in the lower well is omitted. Two different methods have been described based on the type of filter used between the two wells:³¹

Method 1 In the leading front method (Fig. 141.2) a 150 μm thick cellulose nitrate filter is used. The chamber

FIGURE 141.2 Evaluation of chemotaxis in a Boyden chamber, using the leading front method. Neutrophils migrate from the upper to the lower well (toward a chemoattractant) through a cellulose nitrate filter. The distance travelled by the cells is evaluated after staining the filter, using the fine adjustment on the microscope.



is incubated for 30 min at 37°C in humidified, 5% CO₂ atmosphere, and then the filter is removed, fixed, stained, dehydrated, clarified in xylene, and mounted on a glass slide. Cellular movement is evaluated by measuring the distance between the top of the filter and the leading front, where the two last cells are in focus.

Method 2 In the lower surface count method a thin (12 μm) polycarbonate filter with a pore size of 2.0 μm diameter is used. After incubation the filter is removed and the number of cells that pass through the filter is enumerated in the lower well, using a cell counter or a spectrophotometric method.⁸ Cells attached to the lower surface of the filter are counted after wiping the upper surface of the filter and staining with Wright's stain.

These two methods provide different data on neutrophil chemotaxis but, according to some investigators, both methods are strongly influenced by the degree of adherence and gravity.

Thus, assays to evaluate chemotaxis in a collagen gel matrix⁵ or under-agarose¹⁷ have been developed. The collagen gel matrix assay consists of a visual assay obtained by two capillary tubes with their ends covered by a dialysis membrane and connected by a polyethylene tubing sleeve. The compartment formed between the ends of the two tubes is filled with a solution of monomeric collagen and put under a microscope. Cell movements are recorded microscopically after addition of cell suspension and chemoattractant. This technique is quite complex but it allows study of neutrophil movement step-by-step in a microenvironment similar to an *in vivo* system.

The under-agarose technique is a quite simple, inexpensive and widely used method based on the preparation of a gel matrix in a teflon chamber. Wells are cut in the gel and cell suspensions are added to the wells. Different chemoattractants can be put in separate wells and in some cases chemoattractant is omitted in order to evaluate spontaneous motility. After incubation, agarose is flooded with methanol, followed by 35% formaldehyde in order to harden the gel and, after staining, the migration distance run by neutrophils toward the chemoattractant well is evaluated by microscopy.

Finally, some authors²⁴ have suggested a more complex method that requires a culture system made of successive layers of collagen, fibroblasts, collagen, and a confluent monolayer of epithelial cells coated on a porous membrane. Cells are put at the basal side of the membrane and cells passing over the epithelial layer towards chemoattractant stimuli are counted. This technique is particularly useful for studying chemotaxis and diapedesis of neutrophils during infection in the mammary gland, but it is quite difficult to perform and to standardize.

Adherence

Assays for evaluation of adhesive capability of neutrophils could be schematically divided into two groups: (1) those evaluating adherence to inert substances, such as polysaccharides, nylon fibers, glass, or plastic, and

(2) those that measure adhesiveness to biological structures, such as endothelial cells, monolayers, or cell culture systems. The latter assays reproduce interactions between neutrophils and biological barriers in a more complex way, similar to that which occurs *in vivo*. However, they are strongly influenced by extragranulocytic factors; thus most of these techniques are difficult to standardize and lack reproducibility.

Techniques that evaluate adherence to inorganic substances have been developed using column or microtiter plates. Briefly a borosilicate glass column can be filled with packed Sephadex or nylon fibers and neutrophil suspensions are percolated through the column. The percentage of adhered cells is then calculated by counting and subtracting the recovered cells. Different stimulators or substances can be used. All of these methods lack precision; thus it is strongly recommended that these tests be done in triplicate. Assays in microtiter plates are performed by adding suspensions of neutrophils in flat bottom wells together with different activators or inhibitors, and they are useful for testing the influence of different substances on neutrophil adhesiveness. Nonspecific adherence to plastic wells or adherence after activation with an activation agent (e.g. phorbol myristate acetate [PMA] or zymosan activated serum [ZAS]) can be evaluated. In order to remove nonspecific activation due to contact with plastic wells, pre-coating of some wells with gelatin can be performed. After incubation and washing to remove non-adherent cells, the percentage of adhered cells is evaluated by microscopic count using an inverted microscope. Otherwise neutrophils can be lysed and indirectly evaluated by means of chemical or fluorometric methods. Fluorometric labelling with calcein¹⁶ or colorimetric methods to quantify myeloperoxidase or acid phosphatase are commonly used.²² Results should be reported as the number of adhered cells compared to a standard curve obtained by different dilutions of neutrophils from the same animal. Assays in microtiter plates are more reproducible compared with those in columns and require smaller amounts of blood; thus they are more applicable for small animals.

Many assays have been reported to test adhesive capability of neutrophils to cell layers or tissues. The simplest methods use cultured endothelial cells packed into an adherence column instead of nylon fibers or Sephadex. Other techniques using monolayer of cultured cells of different origin (endothelial, fibroblasts, myocytes) have been reported. Target cells may be isolated from fresh tissues and cultured on a coverslip until confluent. An adhesion assay chamber is obtained by adding a second coverslip separated from the monolayer by a rubber O-ring. Neutrophils are added in this incubation chamber and adhesion is evaluated by a visual method, using an inverted microscope equipped with phase-contrast optics. The number of neutrophils remaining on the slide, after washing away the non-adherent cells, is enumerated. This technique reproduces the complexity of interactions between neutrophils and tissue cells and is very useful for studying the pro-

duction of cytokines by endothelial or mesenchymal cells, or the mechanism of interaction between neutrophil adhesion molecules and their ligands. However, the technique is difficult to perform, lacks reproducibility, and is influenced by extra-neutrophilic factors.

Particle Uptake

The capability of neutrophils to phagocytize particles can be evaluated by visual, spectrophotometric or flow cytometric assays. Many different particles can be used, including latex beads, yeasts (*Saccharomyces* or *Candida*, for instance), and bacteria (*Escherichia coli* or *Staphylococcus aureus*). Particles are incubated on a glass slide with a suspension of isolated neutrophils; then ingestion is stopped by adding an ice-cold isotonic solution, and phagocytosis is evaluated by various methods. The simplest method is microscopic evaluation of the percentage of cells that have phagocytized particles, after staining adherent cells. In some assays, particles can be pre-labelled with a fluorescent dye (fluorescein isothiocyanate or acridine orange) and the mean number of ingested particles per neutrophil determined using a fluorescence microscope. Fluorescence of non-ingested particles is inactivated by treating the cellular suspension with trypan blue solution.³⁴ The main problem with this technique is that results are strongly affected by adherence to the glass slides; therefore assays using neutrophil suspensions in tubes or flasks have been developed.²⁷

Another interesting technique uses polystyrene latex beads (1.09 μm diameter).²¹ After incubation, non-ingested particles are removed by centrifugation and the supernatant is discarded; then neutrophils are ruptured and ingested latex particles are solubilized with 1,4-dioxan. The amount of latex is indirectly determined in a spectrophotometer at 255 nm. This method is very simple, inexpensive and rapid, but it does not permit determination of the percentage of neutrophils phagocytizing particles.

Finally, in some assays ingestion has been evaluated after labeling bacteria with radioactive materials followed by evaluation of the quantity of radioactivity in cell suspension.¹⁹ However, this technique is very complex and expensive and requires handling of radioactive materials.

Killing

Many assays have been developed to evaluate bactericidal activity of neutrophils. These have been divided into three groups:

Group 1 This group includes techniques that evaluate residual viability of bacteria incubated with neutrophil suspensions. These assays are easy to perform and can be applied to whole blood. The method consists of incubation of a suspension of live bacteria with neutrophil suspensions and serum in order to allow opsonization. After incubation, bactericidal activity is determined by counting colony-forming units, after cultivation in blood agar plates, or by enumerating bacteria using a

spectrophotometric assay after addition of a tetrazolium compound.²⁸ Live bacteria reduce tetrazolium to red-purple formazan while dead bacteria do not. Results are compared to a standard curve obtained with a known number of live bacteria.

Group 2 This group includes techniques that quantify neutrophil lysosomal enzymes. Many assays have been developed to quantify activity of lysosomal enzymes (myeloperoxidase, lysozyme, lactoferrin, cationic proteins, neutral proteases, acid hydrolases, and proteases) after neutrophil lysis. Most of them utilize colorimetric or ELISA methods that can be performed using a spectrophotometer or a microtiter plate reader.

Group 3 This group includes techniques that quantify the production of oxygen compounds produced by respiratory burst. Neutrophils generate reactive oxygen species (ROS) that play an important role in bactericidal activity. Many ROS are involved but superoxide anion is the precursor. Several assays have been developed to test the oxidative burst of neutrophils. Most of them are performed in multiwell microplates. Neutrophil suspensions are incubated in the presence of activators (such as PMA or zymosan) and after incubation, different methods to measure ROS production can be used. Intracellular and extracellular ROS production can be made by pre-treating cell suspensions with the NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI) or with superoxide dismutase (SOD) or catalase (CAT), which are superoxide and hydrogen peroxide scavengers, respectively. These substances do not cross cell membranes; therefore they inhibit only extracellular ROS.

A rapid assay to detect respiratory burst activity evaluates the capability of neutrophils to reduce nitroblue tetrazolium salt (NBT) to formazan with production of a reddish-purple color that can be quantitated spectrophotometrically.¹⁴ Recently, a microplate assay to measure ROS production in bovine neutrophils has been described.¹⁸

In the cytochrome *c* reduction assay, ferricytochrome *c* is added to wells and is reduced by superoxide. Changes in absorbance at 550 nm are spectrophotometrically evaluated at 10 minute intervals. This assay is reported to be a specific indicator of extracellular superoxide production.

Chemiluminescence can be evaluated by addition of methyl cypridina luciferin analog (MCLA). After addition of MCLA, decrease of chemiluminescence is measured at 10 minute intervals using a microplate luminometer. The assay is reported to be a specific indicator of superoxide production.

A commercially available test (Amplex-Red-dependent fluorescence) consists of a reaction mixture (resorufin and horseradish peroxidase) that is added to neutrophils. Samples are evaluated in a fluorimeter at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. The test detects extracellular hydrogen peroxide. Other fluorescent assays include the dihydroethidium (DHE) and dichlorodihydrofluorescein diacetate (H2-DCFDA) fluorescence assay, and the luminol and isoluminol chemiluminescence assay.

All of these assays are suitable for detection of neutrophil oxidative burst in the bovine. Intra-assay repeatability is good; however, variability is high. The luminol assay has the lowest interassay coefficient of variation (CV) while other tests such as cytochrome *c* reduction, Amplex Red and DHE have high interassay variability (CV > 30%).

In dogs, studies using evaluation of ROS production using cytochrome *c* reduction,² luminol chemiluminescence,³⁴ H₂-DCFDA,²⁰ and NBT reduction⁷ have been reported.

FLOW CYTOMETRIC TESTS FOR NEUTROPHIL FUNCTION

The previously described measurements of neutrophil function using physical, microscopic, or chemical methods remain time-consuming. Moreover, because phagocytosis is due to sequential activation of multiple steps (rearrangement of cytoskeleton, chemotaxis and adherence, degranulation, and production of oxygen radicals) and alteration in any single step could influence the whole process, a panel of assays is usually applied. This requires large numbers of neutrophils that are difficult to obtain from small or diseased animals. Flow cytometric assays for testing neutrophil function have significant advantages when compared to classical ones: they are very rapid to perform, require small numbers of cells (at least 50–100 times fewer than conventional assays) and, using gating techniques, can be applied on heterogeneous populations of cells such as whole blood, avoiding neutrophil isolation that could induce significant activation of cells. Flow cytometry measures fluorescence and light scatter signals through a laser source (see Chapter 137). Using gating and multicolor techniques, cell populations can be resolved and analysis restricted only to cells of interest. Many different flow cytometric assays have been described in human medicine³⁵ but only a limited number have been validated and utilized in veterinary medicine.

Cytoskeletal Actin

Actin is a major cytoskeletal protein of neutrophils and can be found in a globular monomeric form (G-actin) and a filamentous polymeric form (F-actin). During activation, G-actin changes into F-actin and accumulates at the periphery of the cells. This process is involved in adhesion, deformability, and cellular movements. Dysfunctions in actin dynamic polymerization have been linked to susceptibility to infectious diseases.

Flow cytometric detection of F-actin can be assessed using the fluorochrome NBD-phalloidin. Whole blood cells are activated, then cells are fixed with 3% paraformaldehyde and RBCs are lysed. Leukocytes are permeabilized and stained with fluorochromes. In the flow cytometer, fluorochromes within neutrophils are excited with standard laser light and emit green fluorescence. The amount of fluorescence is correlated with F-actin content. This technique has been used in cattle, where

increases of F-actin were found after neutrophil activation.¹²

Surface Adhesion Molecules

Flow cytometric assays for neutrophil adhesion are mainly based on detection of adhesion molecules on the cellular surface. Adhesion molecules may be divided into two groups: the selectin family that establishes a weak association of neutrophil with endothelial cells, and the integrin family that interacts with the immunoglobulin superfamily on endothelial cells, resulting in a firm bond that allows neutrophil diapedesis. The expression of adhesion molecules can be readily quantified using monoclonal specific antibodies. Whole blood is preferable in order to avoid neutrophil activation during the isolation technique. After labeling cells with fluorochrome-conjugated antibodies, the expression of membrane surface molecules is evaluated on gated neutrophils and expressed as mean (or median) fluorescence channel units. Integrins increase on neutrophil cell membranes after activation due to externalization of adhesion molecules from cytoplasmic granules (Fig. 141.3). On the contrary, expression of selectins on the cell membrane of activated cells is controversial.

This assay is simple and rapid but requires careful standardization of procedures, number of cells, and titration in order to work with a large excess of antibody. In veterinary medicine many studies using this technique have been reported, with particular attention given to canine and bovine adhesion molecules.^{3,10,15,30,32,33}

Phagocytosis

Many different assays to quantify neutrophil phagocytic capabilities have been described. These methods entail incubating whole blood with fluorescently conjugated particles of different origin (inorganic beads, bacteria, yeast). The amount of fluorescence is taken as an

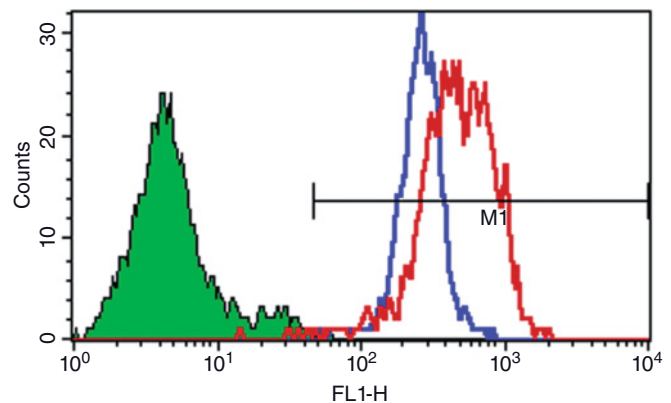


FIGURE 141.3 Flow cytometric evaluation of membrane integrin CD11b in bovine neutrophils. LPS-activated neutrophils (red line) express increased fluorescence compared to non-activated neutrophils (blue line). Shaded line indicates isotypic control.

indicator of phagocytic activity (Fig. 141.4). Heparinized whole blood is used because other anticoagulants chelate Ca^{2+} that is necessary for phagocytosis. A major limitation is that nonspecific adherence to the neutrophil membrane can cause an overestimation of phagocytosis. This aspect could be partially limited by quenching fluorescence of non-phagocytized particles using a quenching solution such as trypan blue or crystal violet. Alternatively, a control sample can be incubated at 4°C that inhibits phagocytosis and allows quantitation of nonspecific adherence.

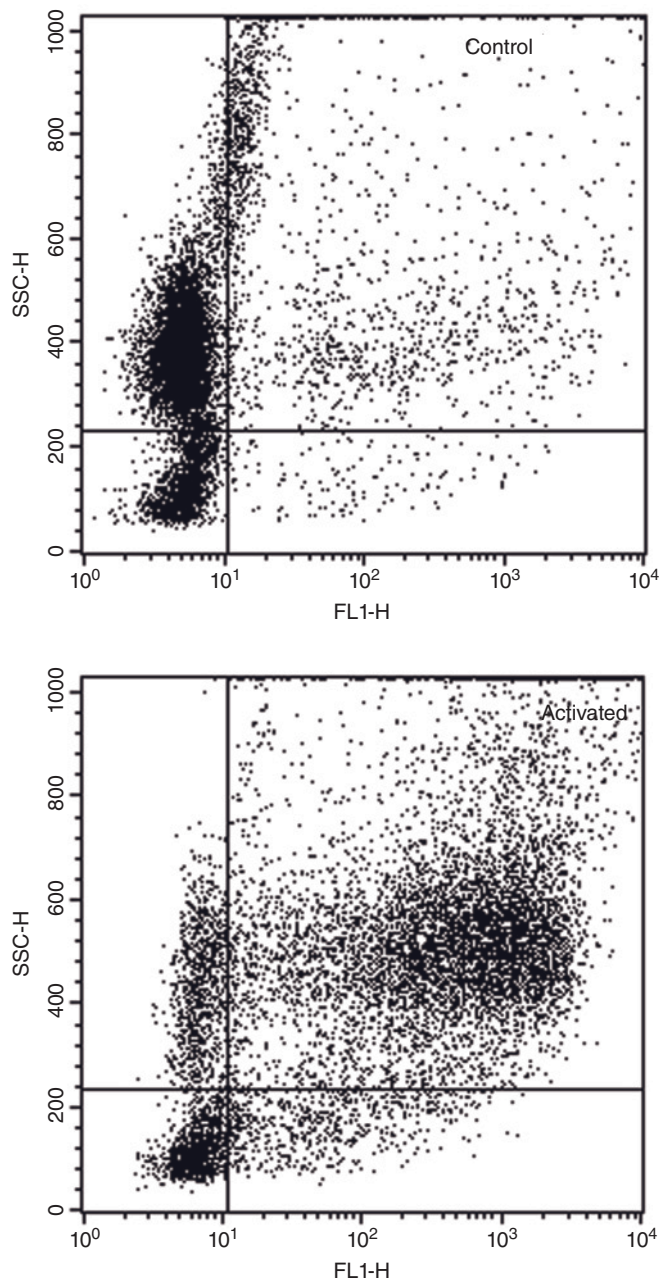


FIGURE 141.4 Flow cytometric evaluation of phagocytosis of fluorescein-labeled *Escherichia coli*. A high percentage of neutrophils shows fluorescence due to bacterial ingestion. In the control sample, phagocytosis is inhibited by incubating cells at 4°C .

An interesting variant of this method allowing simultaneous evaluation of phagocytosis and oxidative burst has been described in cows,²³ dogs, cats, and horses. The technique couples evaluation of ROS production using a green fluorescent dye (dihydrorhodamine 123 or 2,7-dichlorofluorescein [DCF]) with ingestion of propidium-conjugated bacteria that emit a red fluorescence. A double color analysis allows easy measurement of particle uptake and oxidative burst activity simultaneously. Recently, commercially available flow cytometric assays have been validated for the bovine species, and show good agreement with other classical methods.⁹

Reactive Oxygen Species Production

Many flow cytometric tests to measure ROS production have been developed. One of the most frequently used is the DCF assay. The acetate form enters the cells and is oxidized mainly by hydrogen peroxide giving a green fluorescence. A similar technique uses dihydrorhodamine 123 which is converted to the fluorescent rhodamine 123. The benefits of flow cytometric techniques versus conventional ones are that they are rapid, they use small sample volumes, and whole blood samples can be used. However, eosinophils, that are not differentiated based on scatter properties, are capable of an oxidative burst; thus results on whole blood must be carefully evaluated in animals with hyper eosinophilia.

APOPTOSIS

The study of neutrophil apoptosis, or programmed cell death, is another challenging area of neutrophil function. Neutrophils are characterized by a very short lifespan. Evaluation of neutrophil apoptosis is interesting because many different cytokines and also substances produced by pathogens may influence the rate at which neutrophils undergo apoptosis and/or necrosis.

Many assays, using conventional and flow cytometric techniques, have been developed to test neutrophil apoptosis. Conventional techniques mainly use cytochemical stains or colorimetric evaluation of proteases involved in the apoptosis process (i.e. caspase-3). Flow cytometric techniques have the advantage of assessing apoptosis in a large number of cells and are rapid and easy to perform. Many different assays have been developed that can evaluate the following:

1. Scatter properties Apoptotic cells lose water and decrease granularity. This aspect can be easily identified by decreases in both forward and side scatter. However, this method can not distinguish between apoptotic and necrotic cells.
2. DNA content The content of DNA can be evaluated using the fluorescent dye propidium iodide (or other dyes such as acridine orange, ethidium bromide, Hoechst 33342, or actinomycin D) after cell permeabilization. Apoptosis results in DNA frag-

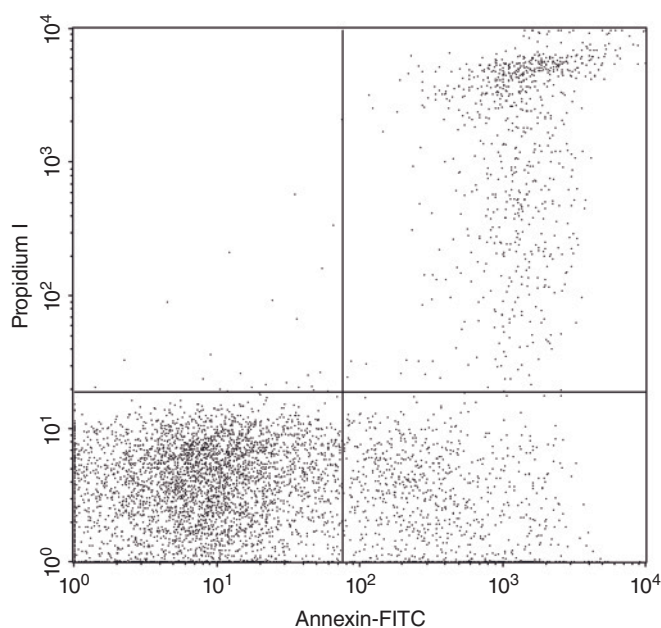


FIGURE 141.5 Flow cytometric evaluation of neutrophil apoptosis using double labeling for annexin V and propidium iodide. Cells in early apoptosis are detectable in the lower right quadrant while necrotic cells are in the upper right quadrant. (Courtesy of M.E. Gelain.)

mentation; thus the detection of cells with less DNA content than normal diploid neutrophils is suggestive of apoptosis or necrotic cells.

- Surface markers of apoptosis Annexin V is a polypeptide that exhibits a strong affinity for phosphatidylserine molecules that are present only on the inner layer of cell membrane in viable cells. During apoptosis phosphatidylserine residues are exposed on the cell membrane. Early apoptotic neutrophils and necrotic cells can be easily detected by double labeling cells with fluorescent-conjugated Annexin V and propidium iodide that enters only necrotic cells (Fig. 141.5).
- Terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) The principle of this assay is that, during apoptosis, terminal deoxyribonucleotidyl transferase (TdT) adds nucleosides to the free 3' ends produced from DNA cleavage. Neutrophils are permeabilized and incubated with biotinylated nucleosides and TdT; after washing fluorescent-conjugated streptavidin that binds to the biotin is added and the number of cells in apoptosis is detected using a fluorescent microscope or flow cytometer. This assay is one of the more widely used methods to detect apoptosis. However, nonspecific positivity in necrotic cells is reported; thus it should be combined with one or more other techniques.

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Evaluation of Platelet Function

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Buccal Mucosal Bleeding Time

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Acronyms and Abbreviations

ADAMTS13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 also known as von Willebrand factor-cleaving protease (VWFCP); ADP, adenosine diphosphate; ATP, adenosine triphosphate; BMBT, buccal mucosal bleeding time; CADP, cartridges coated with collagen and ADP; CEPI, cartridges coated with collagen and epinephrine; CT, closure time; DDAVP, desmopressin acetate (1-(3-mercaptopropionic acid)-8-d-arginine vasopressin monoacetate (salt) trihydrate); FITC, fluorescein-isothiocyanate; GPIb, glycoprotein; GPRP, synthetic peptide Gly-Pro-Arg-Pro; LTA, light-transmission aggregation; MPC, mean platelet component concentration; NSAID, non-steroidal anti-inflammatory drug; OMT, oral mucosal bleeding time; PAF, platelet activating factor; PFA-100®, platelet function analyzer; PPP, platelet-poor plasma; PRP, platelet-rich plasma; SPD, storage pool disease; VWF, von Willebrand factor.

Tests of platelet function have been used in multiple ways in both clinical and research settings. In veterinary medicine they have primarily been used for diagnosis of bleeding disorders, and to predict bleeding risk associated with invasive procedures. More recently, platelet hyperfunction has been found to be an important aspect of many disease processes, and assays that can detect platelet hyperactivity and possibly predict thrombosis have increasingly gained interest. Table 142.1 lists currently available platelet function tests described in this chapter.

This chapter discusses currently available tests of platelet function, their advantages and drawbacks, and their utility in veterinary clinical and research settings. The chapter is focused on tests with published data in the veterinary literature, and does not provide a discussion of all available tests of platelet function.

BUCCAL MUCOSAL BLEEDING TIME

The bleeding time is one of the oldest tests still in clinical use. The buccal or oral mucosal bleeding time (BMBT or OMT) has been the classic *in vivo* test to evaluate primary hemostasis in veterinary medicine (Table 142.2), although other types of bleeding time tests have been evaluated.⁴⁶ The template bleeding time has been reported to have poor reproducibility in horses, with coefficients of variation ranging from 26.8% to 45.5%.⁵² The BMBT or OMT is performed by evaluating hemorrhage from a standardized incision in the oral mucosa using a disposable, single-use, spring-loaded lancet.²¹

The BMBT has been used to screen patients preoperatively,³⁰ to find a cause of bleeding in an actively bleeding patient, to determine if an individual has a

TABLE 142.1 An Alphabetical List of Tests of Platelet Function Described in Chapter 142 With Reference to the Veterinary Literature

Name of Test	Principle	Clinical Applications	Reference
Bleeding time	In vivo cessation of blood flow	Screening POC test	5, 21, 46, 51, 52
Flow cytometry	Measurement of platelet glycoprotein and activation markers by fluorescence	Diagnosis of platelet glycoprotein defects, detection of platelet activation in vivo or in response to agonists	4, 24, 28, 29, 38, 43, 47, 54, 56, 64
Full blood count	Automated impedance or flow cytometry-based analysis of cells	Abnormalities in platelet number, size, and distribution. Platelet density value now available as POC measure of platelet activation	44
Light transmission aggregometry	Low shear platelet-to-platelet aggregation in response to agonists	Diagnosis of a wide variety of acquired and inherited platelet defects	10, 42, 50
PFA-100®	High-shear platelet adhesion and aggregation during formation of a platelet plug	Detection of some inherited and acquired defects in primary hemostasis. Highly dependent on von Willebrand factor	7, 11, 40, 41, 53
Whole blood aggregation	Change in impedance in response to platelet agonists	Diagnosis of a wide variety of acquired and inherited platelet defects	23, 55

TABLE 142.2 Normal Bleeding Times Reported in Different Companion Animal Species

Animal Species	No.	Normal Bleeding Time (seconds)		Method	Reference
		Mean \pm SD	Range		
Dogs	34	157 \pm 29		Buccal mucosal	21
Dogs (Greyhounds)	61	129.5 \pm 44.2		Buccal mucosal	51
Cats	14	114 \pm 30		Oral mucosal	49
Horses	20	342 \pm 114	136–894	Template	52

hereditary primary hemostasis disorder, or to evaluate drug effects.^{2,5,21,49} In dogs with von Willebrand disease, the BMBT is reported to be prolonged 85–100% in affected dogs; however, most of these reports consist of small numbers of cases.^{8,5,21} Administration of desmopressin and cryoprecipitate has been reported to significantly shorten the BMBT, and the test, therefore seems to offer a cost-effective method of treatment evaluation for dogs with von Willebrand disease.^{8,9} The BMBT is also prolonged in dogs with thrombocytopenia, and severe azotemia.²¹ In a series of coagulation factor deficient dogs the BMBT has been reported to be normal.⁵ Considerable variability between BMBT measurements in Greyhounds has been found; for example, values may differ by up to ± 2 minutes.⁵¹ The BMBT has been found to correlate weakly with surgical blood loss in dogs;³⁰ however, its value as a routine preoperative screening test in both human and veterinary medicine is debatable.^{21,31} Clearly, more prospective research involving larger populations of animals affected by primary hemostatic disorders is needed before conclusions can be reached regarding sensitivities and specificities, the biological variability and the use of the BMBT as a preoperative screening tool in veterinary medicine. However, because other more sensitive tests of platelet

function are not widely available, the BMBT may still have a place in veterinary practice as a screening tool for disorders of primary hemostasis.

PLATELET AGGREGATION

Platelet aggregation is the historical “gold standard” for testing platelet function defects, although this position is now being challenged by novel methods. The original platelet aggregation method in platelet-rich plasma (PRP) was described in 1962 by Born, and consisted of an absorptiometer with experiments performed at room temperature.¹ Platelet aggregation is currently being performed in PRP at 37°C using a spectrophotometer, or in whole-blood using an electrical impedance method. The aggregation pattern is classically evaluated in response to addition of an exogenous platelet agonist.

Light-Transmission/Optical Platelet Aggregation

Light-transmission aggregation (LTA) is measured in a spectrophotometer attached to a chart recorder or a computer with relevant software. The instrument is

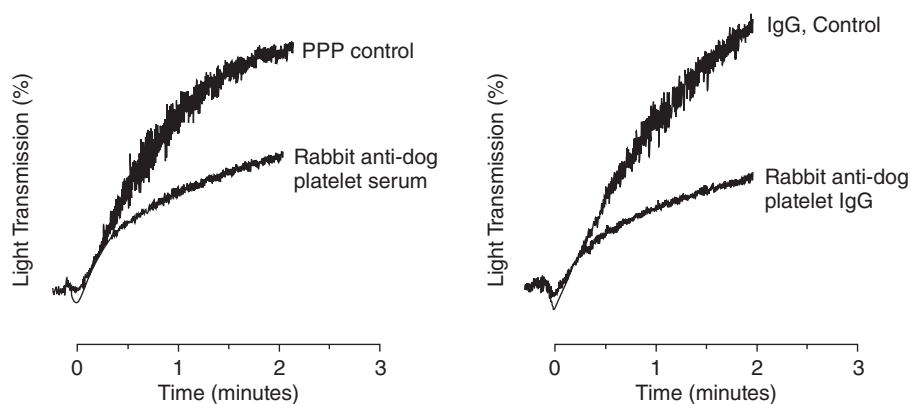


FIGURE 142.1 Aggregation response to thrombin after addition of rabbit anti-dog platelet antiserum and IgG to normal dog platelets. (Reproduced from Kristensen AT, Weiss DJ, Klausner JS. Platelet dysfunction associated with immune-mediated thrombocytopenia in dogs. *J Vet Int Med* 1994;8:323–327, with permission.)

standardized for each subject using PRP set as 0% aggregation and autologous platelet-poor plasma (PPP) set as 100% aggregation. As platelets aggregate in response to the addition of an exogenous platelet agonist, the sample becomes more clear and an increase in light transmission through the test sample is recorded (Fig. 142.1).

Platelet aggregation responses can be evaluated as the percent maximal aggregation response when compared to PPP, or as the rate of aggregation, calculated as the slope of the aggregation curve.

Light-transmission aggregation is a very useful technique for identification and diagnosis of platelet function defects. By using large panels of agonists at various concentrations, it is possible to obtain a large amount of information about many different aspects of platelet function.⁶⁶ However, LTA is relatively nonphysiological because platelets are separated from other blood components and aggregated under low shear conditions during the test—conditions that do not accurately mimic platelet adhesion, activation, and aggregation in damaged vessel walls.

Light-transmission aggregation can be affected by a variety of analytical and preanalytical variables. Venipuncture should be performed carefully using a 19- to 21-gauge needle and a central vein (e.g. jugular) with minimal stasis. Some laboratories do not consider evacuated collection tube collected blood as suitable for aggregation measurements due to *ex vivo* artifactual activation of platelets by the shear force of the vacuum. However, other laboratories use these tubes without causing significant *ex vivo* platelet activation.³⁷ Sodium citrate (0.102 mol/L, 0.129 mol/L, buffered and non-buffered) at a ratio of nine parts blood to one part anticoagulant is the typical anticoagulant for platelet aggregation testing. Very low hematocrit values can affect aggregation values, as the final plasma concentration of the anticoagulant will be low compared to samples with normal hematocrit, with a higher concentration of free calcium available in the plasma.¹⁵

There are a variety of opinions on whether or not it is necessary or beneficial to standardize the platelet count of the PRP used in platelet aggregation assays.^{33,63} Some report that aggregation responses can vary in

relation to platelet count, and it is recommended that platelet counts be standardized.²⁰ However, mixing PPP with PRP could influence aggregation results because PPP may contain substances released from platelets and other blood cells during the centrifugation process that may influence platelet function.³²

Impedance/Whole Blood Platelet Aggregation

Platelet aggregation may be carried out in a whole blood system using electrical impedance. The impedance method can be used with either PRP or whole blood, although the whole blood method is the most widely used. This method measures an increase in impedance across two electrodes placed in the anticoagulated blood as activated platelets accumulate on them.¹⁹ Whole-blood aggregation has many advantages, including use of smaller blood volumes, immediate analysis of samples without manipulation, and no loss of platelet sub-populations or platelet activation during centrifugation.

Platelet Agonists

Adenosine Diphosphate

Adenosine diphosphate (ADP) is a weak platelet agonist, and was the first known low-molecular-weight platelet aggregating agent. In human platelets, it induces shape change and reversible aggregation. The secondary aggregation sometimes observed after stimulation of PRP with ADP is caused by secretion of dense granule constituents triggered by synthesis of thromboxane A_2 . Although a weak agonist in itself, ADP plays a key role in platelet function by amplifying platelet responses induced by other platelet agonists.

There is considerable interspecies difference in platelet aggregation responses to various agonists; however, platelets from most species appear to respond to stimulation by ADP at concentrations between 5 and 25 $\mu\text{mol/L}$.^{42,50,55} One exception is in canine whole blood, where ADP does not give consistent aggregation responses, possibly due to degradation of ADP by red blood cells and white blood cells.^{23,45}

Collagen

Collagen, either of bovine or equine tendon origin, is typically the strongest of the agonists used in clinical laboratories. Collagen-induced platelet aggregation usually has a lag phase, during which the platelets adhere to the collagen fibrils and undergo shape change and release granule contents. Canine platelets have been reported to respond in a dose-dependent manner to 1–20 µg/mL collagen.^{23,42} Platelets from horses, cows, pigs, buffalo, and sheep all responded to collagen in one study, with equine platelets being the most sensitive to collagen.⁵⁰

Arachidonic Acid

Arachidonic acid is converted to thromboxane A₂, a potent platelet agonist, when reacting with cyclooxygenase. The aggregation response induced by arachidonic acid is completely inhibited by aspirin in humans. There are reports of variable platelet responses to arachidonic acid in the veterinary literature, and this agonist may, therefore not be of as great value for testing aspirin and other non-steroidal anti-inflammatory drug (NSAID) effects on animal platelets.^{10,23,50} Pelagalli⁵⁰ showed that arachidonic acid was less effective than ADP, collagen, or platelet activating factor (PAF) in inducing aggregation of PRP from horses, buffaloes, pigs, and sheep, although a maximal aggregation response was achieved at 7.5 mmol/L. The use of arachidonic acid as an agonist in canine platelet aggregation experiments has been controversial. Jüttner²³ found no response to arachidonic acid at 0.5 mmol/L and 1 mmol/L in any dogs tested, whereas others have found that canine platelets aggregate in response to arachidonic acid.^{13,55} One study found that the variable platelet responses to arachidonic acid were breed-dependent,¹⁰ and it has been suggested that the differences in sensitivity to arachidonic acid in dogs may be because of inherited differences in intracellular signaling pathways.²²

Epinephrine

Epinephrine is the most erratic and unreliable of the agonists for platelet aggregation. Epinephrine is usually regarded as ineffective by itself as an aggregation initiator, but has been used to enhance aggregation responses of other platelet agonists.

Thrombin

Thrombin is a very potent platelet agonist and in addition cleaves fibrinogen to form a fibrin clot in plasma, which makes it a very difficult agonist to use for platelet aggregation testing. The synthetic peptide, Gly-Pro-Arg-Pro (GPRP), inhibits thrombin-induced fibrin polymerization while not inhibiting platelet aggregation responses.⁴²

Platelet Secretion Techniques

Platelet dense granule release can be evaluated in a specialized aggregometer, called a lumiaggregometer, simultaneously with the aggregation response. Lumiaggregation is based on the conversion of ADP, released from platelet dense granules, to ATP. This ATP then reacts with the firefly lantern extracts (luciferin and luciferase), generating adenylyl-luciferon. Light is emitted when oxidation of adenylyl-luciferon occurs. An ATP standard is used to calibrate the signal. Studies of platelet secretion allow the assessment not only of platelet aggregation but also of granule secretion. This is useful in the diagnosis of platelet disorders such as storage pool disease and release defects, especially in cases where animals have clinical bleeding with normal aggregation tracings.⁶³

Platelet Aggregation in Clinical Disorders

Platelet aggregation has been used in the evaluation of both inherited and acquired diseases. Animals with Glanzmann thrombasthenia have a defect involving glycoprotein complex IIb-IIIa (integrin αIIbβ₃). The integrin αIIbβ₃ is required for platelet aggregation, and the diagnostic features of Glanzmann thrombasthenia are absent aggregation to virtually all physiologic agonists, and an abnormal clot retraction.³ Storage pool disease (SPD) is characterized by defective secretion of adenine nucleotides. Animals with dense-granule SPD may have impaired platelet aggregation response to ADP and collagen or normal platelet aggregation.⁶ The SPD associated with Chediak-Higashi disease causes impaired platelet aggregation response to collagen and a virtually absent release of adenine nucleotides, measured by lumiaggregation.¹⁸ See Chapter 82 for a more detailed description of inherited platelet disorders.

Acquired platelet function defects detected by platelet aggregation have been described in uremia, immune-mediated thrombocytopenia, laminitis, infectious diseases, malignancies, heart disease, and drug-related disorders.^{25,42,45,48,58} These disorders are discussed in detail in Chapter 83.

PLATELET FUNCTION ANALYZER – PFA100®

Because of several disadvantages of the bleeding time as a screening test for platelet dysfunction in clinical practice, several newer alternative technologies have been developed. The most widely used in veterinary medicine is the platelet function analyzer (PFA)-100® (Dade-Behring, Deerfield, IL). The test is a modified bleeding time and is based on the three phases of platelet function: adhesion, aggregation, and secretion.

Test Principle

The PFA-100 test is performed by pipeting 0.8 mL of citrated whole blood into a disposable cartridge. The blood sample is then drawn, under a constant vacuum,



FIGURE 142.2 The PFA-100® instrument (Dade-Behring).

through a capillary tube and an aperture 150 μm in diameter. The membrane is coated with collagen and epinephrine (CEPI) or collagen and ADP (CADP). Activation of platelets and adhesion to the membrane is caused by the high shear rates (4000–5000 s^{-1}) generated in the PFA-100 system and is mediated via a platelet surface glycoprotein Ib-von Willebrand factor (GPIb-VWF) interaction. Subsequent release of platelet granule contents and the presence of collagen and epinephrine or ADP on the membrane result in platelet aggregation. When the aperture is occluded by aggregated platelets, blood flow through the membrane stops. This is referred to as the closure time (CT) (Fig. 142.2).²⁶ Normal reference intervals for CT have been reported to be 53–98 seconds for CADP-CT and 92 to >300 seconds for CEPI-CT in dogs,⁴⁰ and 61–116 seconds for CADP-CT and 159 to >300 seconds for CEPI-CT in horses.⁵³ It is generally recommended that each laboratory establishes its own normal range for CT in both cartridge systems of the PFA-100. Ideally, normality should be established by parallel platelet aggregation studies and measurement of VWF activity. The normal range must be established using the same concentration of sodium citrate that is routinely used in the institution (see below).

Factors Affecting Closure Time

PFA-100 CTs are sensitive to both platelet number and function. In dogs there is an approximate linear

relationship between CT and platelet count below $150 \times 10^9/\text{L}$.⁴¹ However, no association has been found between platelet count and CT in Cavalier King Charles Spaniels with idiopathic inherited macrothrombocytopenia, probably reflecting a normal total circulating platelet volume in these dogs.⁵⁹

Closure time also increases with decreasing hematocrit in humans and dogs.^{27,41} The exact threshold at which the CT is affected will probably vary between species. In dogs a prolongation of the CT is seen at hematocrit values below 30–40%.^{7,41} Plasma levels of VWF are an important determinant of CT in the PFA-100, because platelet aggregation at high shear rates is highly dependent on VWF. In humans, it has recently been shown that plasma VWF levels modulate PFA-100 CT to a greater extent than intrinsic platelet function.¹⁶ An association between PFA-100 CT and VWF has been shown in healthy dogs,⁵⁷ and in dogs with inherited,^{7,41} and acquired⁵⁸ von Willebrand disease.

The concentration of anticoagulant is an important determinant of CT in humans and dogs. Test times are longer in 0.129 mol/L (3.8%) citrate than in 0.109 mol/L (3.2%), probably due to greater calcium chelation. In dogs, the PFA-100 CT appears to be stable up to 8 hours after the blood sample is collected,⁴⁰ although testing within 2 hours is generally recommended. The manufacturer of the PFA-100 currently recommends testing within 4 hours for humans.

Detection of Platelet Function Defects

The PFA-100 system has been designed for use in humans, and there has been some concern about use of the CEPI cartridge in veterinary medicine. Very long CTs and non-closure (>300 seconds) have been reported in a substantial proportion of healthy, unmedicated dogs and horses.^{7,41,53,59} It has been suggested that subjects with CEPI-CT > 300 seconds may have an aspirin-like defect; however, normal plasma thromboxane B_2 levels have been demonstrated in dogs with CEPI-CT > 300 seconds.⁴⁵ Epinephrine does not consistently cause platelet aggregation in dogs,¹⁰ whereas in humans epinephrine causes primary aggregation, and this is the most likely cause for the apparently false-positive CEPI-CTs found in dogs and horses.

Hypofunction

The sensitivity of the PFA-100 for detecting von Willebrand disease in humans is very high.¹² In two small case studies of dogs with von Willebrand's disease (see Chapter 81), all affected dogs had prolonged CADP-CT, suggesting a similar high sensitivity.^{7,41} Callan and Giger⁷ have reported a sensitivity of 95.7% and specificity of 100% for detecting primary hemostatic disorders (thrombocytopenia, thrombopathia, and type 1 von Willebrand disease) using the CADP cartridge; sensitivity was 95.7% and specificity 82.8% using the CEPI cartridge. The PFA-100 has utility in monitoring responses to DDAVP in dogs with type 1 von Willebrand's disease, where partial correction of

CT typically is obtained. DDAVP administration has been found to significantly shorten the CADP-CT but not the CEPI-CT. The PFA-100 CADP cartridge, therefore appears to be useful in monitoring the response.⁸

Very few studies have documented the utility of the PFA-100 in detecting congenital platelet function disorders in animals. Callan and Giger⁷ found that five out of six dogs with unspecified thrombopathia had prolonged CADP-CT, and a case report has described prolonged CADP-CT in a horse with Glanzmann thrombasthenia.³⁵ In humans, the PFA-100 is sensitive to the presence of Glanzmann thrombasthenia (GPIIb-IIIa deficiency), Bernard-Soulier syndrome (GPIb-IX-V deficiency), and patients with δ -storage pool deficiency and primary secretion defects.¹⁴ However, the International Society of Thrombosis and Haemostasis working group on the PFA-100 has recently recommended use of the PFA-100 be restricted to research studies and prospective clinical trials, due to insufficient sensitivity or specificity.¹⁷

Assessment of acquired platelet dysfunction by the PFA-100 has to some extent been described in veterinary medicine. Both the CADP-CT and CEPI-CT appear to be prolonged by acetylsalicylic acid in dogs and horses.^{41,53} This is in contrast to humans, where the CT will be prolonged with the CEPI cartridge, but normal (or near-normal) with the CADP cartridge.³⁶ Dogs experimentally loaded with endotoxin had shortened CT at 0.5 hours and prolonged CT 48 hours after inoculation. The initial shortened CT may reflect hemoconcentration more than actual platelet hyperfunction.⁶⁵ Dogs with heart diseases causing high-velocity blood flow (mitral valve regurgitation and subaortic stenosis) and an acquired type 2A von Willebrand disease have prolonged CADP and CEPI CT (Fig. 142.3). The mechanism has been suggested to be enhanced cleavage of VWF high-molecular weight multimers by ADAMTS13, a plasma metalloprotease acting on VWF preferentially under conditions of high fluid shear stress.^{57,58} A similar acquired defect with prolonged CT has been found in humans with aortic stenosis.⁶⁰

Hyperfunction

It is unclear whether shortening of the PFA-100 CT truly reflects platelet hyperfunction/increased activity, because there is lack of data that links shortened CT to clinical evidence of thrombosis or thrombosis-related outcome. Shorter CTs have been associated with ST elevation myocardial infarcts in human patients, but data are lacking in veterinary medicine. Greyhounds have been found to have shorter CT compared to other dogs; however, this could be linked to the higher hematocrit found in this breed.¹¹

FLOW CYTOMETRY

Flow cytometry is a versatile tool for study of platelet function, that can be used for multiple purposes in veterinary medicine. Flow cytometry may be used to: (1)

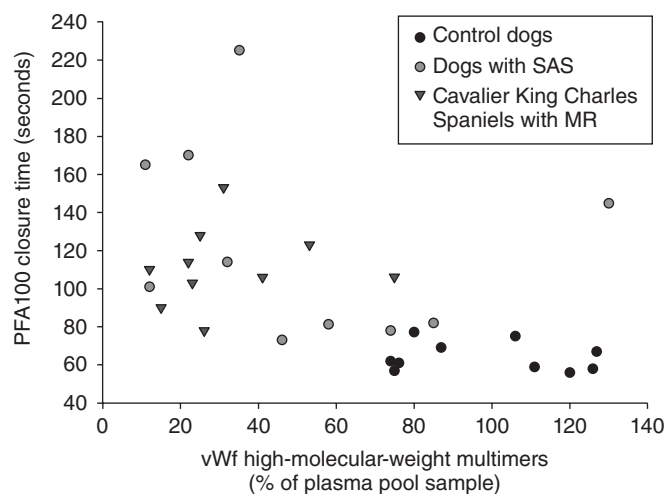


FIGURE 142.3 Platelet function analyser closure times shown as a function of percentage high-molecular-weight von Willebrand Factor multimers (sample relative to pool ratio) (Spearman's rank correlation coefficient; $r = -0.62$, $P = 0.0003$). SAS, Subaortic stenosis, MR, mitral regurgitation. (Reproduced from Tarnow I, Kristensen AT, Olsen LH, et al. Dogs with heart diseases causing turbulent high-velocity blood flow have changes in platelet function and von Willebrand factor multimer distribution. *J Vet Int Med* 2005;19:515–522, with permission.)

measure the activation state of circulating platelets and their reactivity (by activation-dependent changes in the platelet surface, leukocyte-platelet aggregation, and pro-coagulant changes on the platelet surface); (2) diagnose specific platelet function defects (see Chapters 82 and 83); (3) monitor thrombopoiesis by quantifying reticulated platelets; (4) measure platelet count (see Chapter 77); (5) assess anti-platelet drug effects; and (6) perform assays used for research purposes. Only measurement of the activation state of circulating platelets and their reactivity will be described in this section.

Flow cytometry measures specific characteristics of cells as they flow through a flow chamber, and through the focused beam of a laser. The cells can be fluorescently labelled, typically with a fluorescently conjugated antibody, or can be analysed unlabelled. When labelled cells pass through the laser, the laser light activates the fluorophore at the excitation wavelength, and the emitted fluorescence as well as the light scattering properties of each cell are detected. The intensity of the emitted light is directly proportional to the antigen density of the cell (see Chapter 137).

Flow cytometric assays evaluating platelet function can utilize washed platelets, PRP, or whole blood. Use of washed platelets or PRP is potentially susceptible to artifactual *in vitro* activation as a result of separation procedures. Use of whole blood requires a minimum of two antibodies, each conjugated with a different fluorophore: a platelet identifying antibody, to separate platelets from other cells in the blood, together with a "test" antibody recognizing the antigen to be measured. Physiologic or non-physiologic agonists can be used to

assess platelet reactivity. Samples can be stabilized by fixation before or after addition of antibodies. Relevant isotope-matched antibodies are used as negative controls.³⁹

There are many advantages to flow cytometric analysis of platelet function. Both the activation state of circulating platelets and the reactivity of platelets can be assessed; only minute volumes of blood are needed; and the evaluation of function of platelets with severe thrombocytopenia and/or anemia is possible. The application of flow cytometry for evaluating platelet function in veterinary medicine is limited by the lack of commercially available species-specific monoclonal antibodies. Some platelet antigens, (e.g. integrin α IIb β 3), appear to be widely preserved across species, and most anti-human antibodies directed against this antigen are cross-reactive.^{35,54,56}

Measurement of Platelet Activation

The activation state of circulating platelets can be judged by binding of an activation-dependent monoclonal antibody in the absence of an agonist. Inclusion of exogenous agonists allows analysis of the reactivity of platelets *in vitro*. Activation of platelets results in a specific functional response, such as a change in surface expression of a receptor or other antigen or bound ligand. In addition, flow cytometric detection of leukocyte-platelet aggregates and procoagulant platelet-derived microparticles, that are sensitive markers of platelet activation in humans, have been investigated as markers of platelet activation in veterinary medicine (Table 142.3).³⁹

Activation-Dependent Platelet Surface Changes

Markers of platelet activation include activation-dependent conformational changes in integrin α IIb β 3, exposure of granule membrane proteins, such as P-selectin, platelet surface binding of secreted platelet or plasma proteins (i.e. fibrinogen), and development

of a procoagulant surface (assessed by Annexin V binding). The most widely used in veterinary medicine are detection of platelet surface P-selectin expression, binding of fibrinogen, and development of a pro-coagulant surface.

In dogs, horses, and pigs, methods to assess platelet activation by assessing platelet surface P-selectin expression have been described.^{28,38,43,54,56,64} Increased platelet surface P-selectin expression has been demonstrated in dogs with inflammatory disease,⁴⁴ and with immune-mediated hemolytic anemia (see Chapters 33 and 83).⁶¹

Assessment of fibrinogen binding has been described in dogs using a monoclonal antibody that recognizes a receptor-induced binding site (RIBS) on canine fibrinogen.^{4,56} Moreover, exogenous FITC-labelled fibrinogen binding as a marker of platelet activation has been described in dogs.⁵⁶ Binding of an anti-fibrinogen antibody as a marker of platelet activation has been described in horses and pigs.^{24,29,47}

Development of a pro-coagulant surface can be assessed by binding of the protein Annexin V to phosphatidylserine exposed on the platelet surface. This has been described in dogs and horses.^{24,64} Phosphatidylserine exposure combined with bound α -granule proteins and calcium permeability identify coated platelets, a sub-population of platelets that are highly pro-coagulant. Annexin V measurement is independent of species, because it is a placental protein and not an antibody directed against a specific epitope.

Leukocyte-Platelet Aggregates

Leukocyte-platelet aggregates are formed via interaction between P-selectin exposed on the surface of activated platelets and the P-selectin glycoprotein ligand 1 counter receptor on the leukocyte surface.³⁹ Detection of leukocyte-platelet aggregates has been described in dogs (Fig. 142.4) and horses,^{54,56,64} and increased neutrophil-platelet aggregates has been associated with laminitis in ponies.⁶²

TABLE 142.3 Application of Flow Cytometry to the Study of Platelet Activation

Activation-dependent platelet changes	Method	Reference
Surface P-selectin exposure	Binding of monoclonal antibody AC1.2, 1E3, MD6 (dogs) Psel.KO.2.5, SwPsel.1.9 (pigs) CLBThromb/6 (horse)	43, 56, 64 38, 54
Endogenous fibrinogen binding	Binding of monoclonal antibody recognizing receptor-induced binding site on bound fibrinogen	4, 56
Exogenous fibrinogen binding	Binding of an anti-fibrinogen antibody (polyclonal)	24, 29, 47
Development of a procoagulant surface (phosphatidylserine exposure)	Binding of fluorophore labelled species-specific fibrinogen	56
Exposure of lysosomal integral protein	Binding of Annexin V	24, 64
Formation of leukocyte-platelet aggregates	Binding of monoclonal antibody CLBGran/12	64
	Leukocyte-platelet aggregates	64
	Neutrophil-platelet aggregates	56, 62
	Monocyte-platelet aggregates	56
Mean platelet component (automated measurement ADVIA 120)	Refractive index of platelets	44

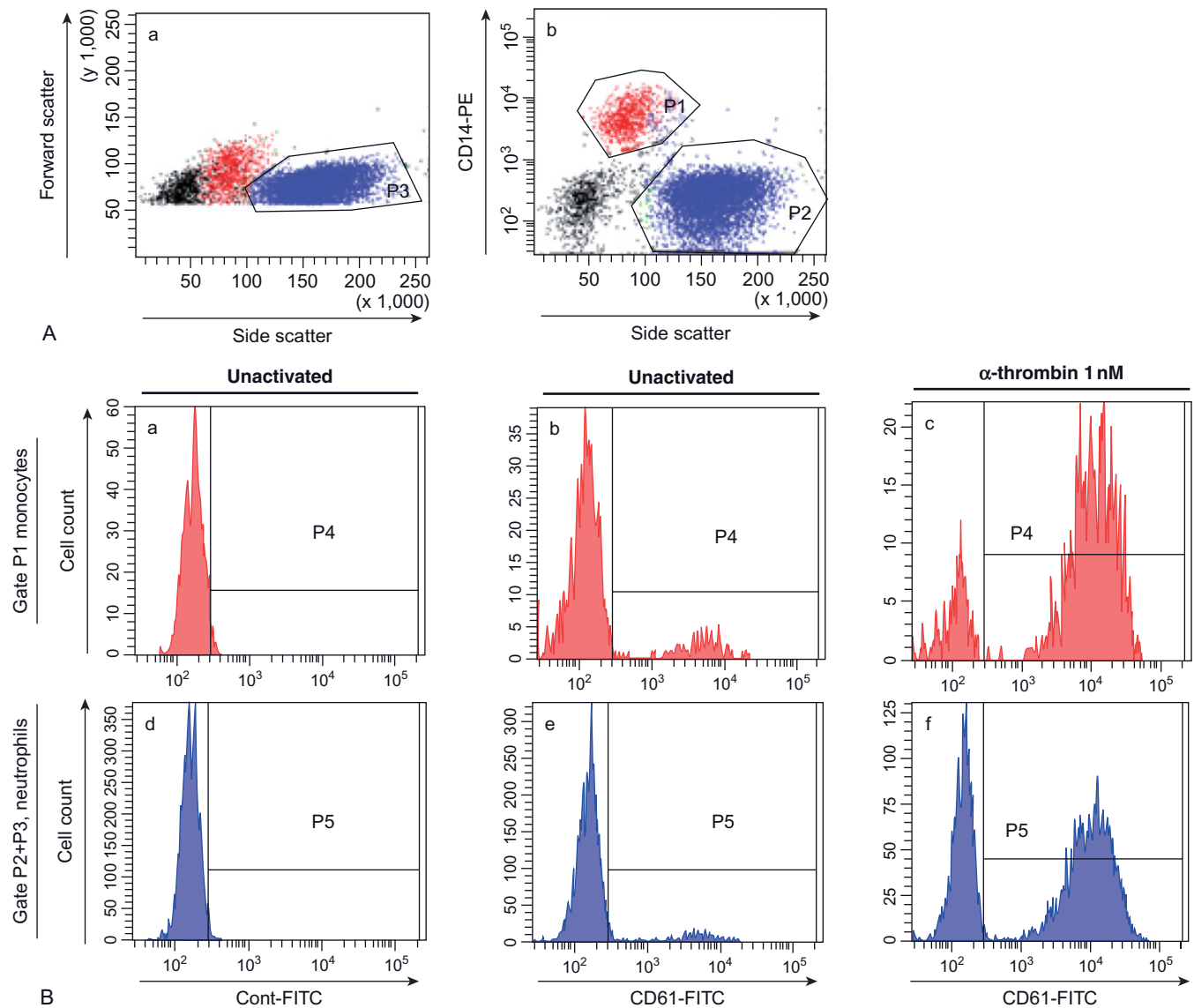


FIGURE 142.4 Flow cytometry gating logic for the detection of leukocyte-platelet aggregates in canine whole blood. (A) Monocytes and neutrophils were gated by a combination of light scattering and CD14-PE fluorescence. Bright CD14 and 90° (side) light scatter define monocytes (P1), dim CD14, forward and 90° light scatter define neutrophils (P2 and P3). (B) Histograms are used to quantify leukocyte-platelet aggregates by percent CD61-FITC positive cells and CD61-FITC mean fluorescent intensity from P1 cells, monocytes (B-a, B-b, B-c) and P2 and P3 cells, neutrophils (B-d, B-e, B-f). Gates P4 and P5 are adjusted such that they include 1% of the isotype-control stained (Cont-FITC) population (B-a, B-d). Unactivated samples had 9.6% monocyte-platelet aggregates (B-b), and 7.5% neutrophil-platelet aggregates (B-e). Activation with 1 nM α -thrombin gave 84.2% (B-c) and 59.7% neutrophil-platelet aggregates (B-f). (Reproduced from Tarnow I, Kristensen AT, Krogh AKR, et al. Effects of physiologic agonists on canine whole blood flow cytometric assays of leukocyte-platelet aggregation and platelet activation. *Vet Immunol Immunopathol* 2008;123:345–352, with permission.)

Automated Measurements

Mean platelet component concentration (MPC) has been investigated as an automated measurement of platelet activity. The MPC estimates the refractive index of platelets. When activated platelets degranulate, the density decreases and the MPC decreases.³⁴ Decreased MPC has been associated with inflammatory disease in dogs, with a significant inverse correlation between platelet surface P-selectin expression and MPC.⁴⁴

FUTURE ASSAYS

Recent abstracts indicate that thromboelastography, including platelet mapping assays, calibrated automated thrombograms, point of care aggregation based assays (VerifyNow, Accumetrics, San Diego, CA), and single platelet detection based assays (PlateletWorks, Helena Laboratories, Beaumont, TX) will likely become future tools for testing the contribution of platelets to the hemostatic process.

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Immunophenotyping and Determination of Clonality

ANNE C. AVERY

Immunophenotyping

- Indications
- Preparation of Samples
 - Blood and bone marrow
 - Aspirates and cavity fluids
- Antibody Panels
- Reporting Flow Cytometry Results
- Reactive Versus Neoplastic
- Lineage Determination
 - Lymphoid or myeloid origin leukemia?
 - Acute or chronic leukemia?
 - Leukemia or lymphoma?

Clonality

- Principles of Testing
- Obtaining and Shipping Samples
- Quality Control
- Sensitivity and Specificity
- Interpretation of Results
- Clonality Testing or Immunophenotyping in Lymphoid Leukemia and Lymphoma – Which to Choose?

Acronyms and Abbreviations

ALL, acute lymphocytic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; ICC, immunocytochemistry; Ig, immunoglobulin; IHC, immunohistochemistry; LGL, large granular lymphocyte; PARR, PCR for antigen receptor rearrangements; PCR, polymerase chain reaction; TCR, T cell receptor.

IMMUNOPHENOTYPING

There are over 40 categories of human lymphomas and leukemias. Although these different subtypes were originally defined by histologic and/or cytologic appearance and clinical behavior, it is now possible to define the majority of these different categories based on the array of proteins expressed on the cell surface using immunophenotyping. Many antibodies are now available for similar work in dogs, and a more limited panel is available for cats.

All immunophenotyping methods utilize monoclonal antibodies specific for cell surface or cytosolic proteins, either directly conjugated to fluorescent proteins or enzymes that catalyze a color change, or detected using secondary reagents specific for the monoclonal antibodies. Immunohistochemistry and immunocytochemistry (IHC and ICC) are used with cells on slides – either paraffin embedded tissue biopsies or cytology preparations. Although IHC is an essential component of evaluating lymph node biopsies, it will not be discussed here. Flow cytometry pro-

vides more flexibility than ICC and will be the focus of this chapter.

Indications

In hematologic malignancy, flow cytometry can be used to accomplish two general goals: to determine if the patient has lymphoma or leukemia, and to determine what type of lymphoma or leukemia is present. Hematologic malignancy can present with a very wide range of clinical symptoms, but flow cytometry is generally indicated in only two: (1) when there are more cells present than are expected (for example lymphocytosis); (2) when there are cells with a cytologically malignant appearance. Table 143.1 gives guidelines for specific situations where flow cytometry may be useful and where it is not likely to be useful. These are just guidelines, however, and as more antibodies become available there may be additional indications. It is also important to contact the laboratory to which you are considering submitting flow cytometry because some

TABLE 143.1 Clinical Indications for Performing Flow Cytometry, and Situations Where Flow Cytometry is Not Informative

	Blood	Masses/Lymph Nodes	Bone Marrow	Cavity Fluids
Indicated	Lymphocytosis Cytologically abnormal cells	Lymphadenopathy Mediastinal masses	Cytologically abnormal cells Increased lymphocyte count	Increased lymphocytes Cells with aberrant morphology suggesting lymphoid or myeloid origin
	Monocytosis	Splenomegaly with cytologic suspicion of lymphoma		
Not indicated	Neutrophilia ^a	Metastatic disease from non-lymphoid tumors ^b	Increased plasma cells or monoclonal gammopathy ^c	Transudates with few cells
	Eosinophilia ^a	Primary tumors of non-lymphoid origin	Loss of a lineage or myelofibrosis	Suspected septic process
	Thrombocytosis	Highly reactive nodes		Suspect carcinoma/reactive mesothelial cells ^b
	Polycythemia Cytopenias			

^aNeutrophils from patients with CML and reactive neutrophilia have the same phenotype. The immunophenotype of canine eosinophils has not been described.

^bThe use of flow cytometry to detect non-lymphoid tumors in lymph nodes and cavity fluids (such as melanoma in a lymph node, carcinoma in a pleural effusion) has not been reported. As antibodies that detect these tumors become available and tested, however, flow cytometry will be an important application for this purpose.

^cThe immunophenotype of canine plasma cells has not yet been described. Therefore, flow cytometry will not be able to distinguish reactive from neoplastic plasma cells (the clonality assay will be useful in these cases).

laboratories may have additional capabilities that have not yet been reported in the literature.

Preparation of Samples

Detection of cell surface antigens by flow cytometry requires cells to be viable when they are stained. Therefore, when cells are prepared steps must be taken to ensure they remain viable until they are processed. Peripheral blood and bone marrow samples can retain reasonable viability for several days when kept refrigerated, but samples from effusions, or aspirates of lymph nodes or masses are less hardy.

The author's laboratory recommends the following procedures be followed when collecting samples for flow cytometry:

Blood and Bone Marrow

Collect these samples in EDTA and ship overnight with a cold pack. Testing within 2–3 days of taking the sample is preferred, but in some cases, blood samples can remain sufficiently viable for 5 days.

Aspirates and Cavity Fluids

It is essential that tissue aspirates and cavity fluids with low protein (<5 mg/dL) be placed in medium with serum in order to preserve viability. Roswell Park Memorial Institute (RPMI) medium, Dulbecco's modified Eagle's medium (DMEM) or similar cell culture media with 10% fetal bovine serum are ideal. This can usually be found in a university setting. In private practices where this is not available, put 0.5–1 mL of 0.9% saline (LRS, Norm R or Norm M may also be used) into

a test tube or red top tube without anticoagulant. Add serum from an animal of the same species (this can be the patient, but it does not have to be) so that the serum is approximately 10% of the total fluid volume. These samples should be shipped with a cold pack overnight and tested as soon as possible. The author has found that this method maintains viability for 2–3 days.

The specific method of preparing cells for analysis differs between laboratories. The simplest is to treat all samples with hypotonic lysing solution to remove erythrocytes and then stain cells immediately. This is the most common method described by the various laboratories which report flow cytometry results.^{24,26–28,31,34,39,50,51,53,54} An alternative is density gradient centrifugation for removing erythrocytes, dead cells and platelets. However, this is more time consuming and may result in selective loss of some cell subsets, and is no longer often used.

Antibody Panels

Each laboratory that offers this test uses different combinations of antibodies, and a different approach to immunophenotyping. The goal of designing an antibody panel is to be able to detect cells with an abnormal phenotype, suggesting malignancy in cases where the diagnosis is not clear, and to determine the lineage of malignant cells. Because there is presently no standardization of antibody panels in either veterinary or human medicine, it seems reasonable to follow the most recent recommendations of a consensus panel on phenotyping human hematopoietic malignancies.^{12,55} Each laboratory should develop its own panels based on the experience of that laboratory, the most current literature, the technology available to that laboratory (the nature of the

flow cytometer) and the array of antibodies available. A minimum panel for canine malignancies should include antibodies to cell surface proteins found on T cells (CD3, CD4 and CD8), B cells (CD21), CD34 (a stem cell antigen that is considered diagnostic for acute leukemia), CD45 (a pan-leukocyte antigen), and CD14 (for identification of monocytes). A detailed description of these antigens is given in Chapter 4. Antibodies to cytoplasmic CD3e and CD79a, which require permeabilization for staining,^{43,47} are essential for determining the phenotype of acute leukemias, because these immature neoplasms often do not express surface antigens. The repertoire of antibodies available for cats is frustratingly limited. Directly conjugated antibodies to CD4, CD5 and CD8 are commercially available, and many anti-B cell antibodies react with feline cells, including anti-canine and anti-human CD21,³² as well as an antibody to murine B220 (A.C. Avery, unpublished observations). In addition, the antibodies used to detect cytoplasmic CD3e¹⁰ and CD79a react with virtually all species, so these could be used in cases of suspected acute leukemia to determine lineage.

It is the author's opinion that the use of commercially available, directly conjugated antibodies is preferable for two reasons. First, when a small number of commercial sources are used, quality control and reagent preparation become less of a variable between laboratories. Second, the use of directly conjugated antibodies, which are more easily prepared by commercial suppliers than individual laboratories, allows for multiparameter flow cytometry, which is essential for identifying cells with an abnormal phenotype. In addition, reagents such as propidium iodide or 7-amino-actinomycin D (7-AAD) to exclude dead cells should always be included in flow cytometry studies when staining for surface antigens, because dead cells can bind antibodies nonspecifically, or are permeable to intracellular staining where some antigens are even in the absence of surface expression.³⁵

Reporting Flow Cytometry Results

Following the lead of consensus recommendations for human lymphoid malignancies,⁵⁵ a useful approach to reporting would include both objective data (percentage of cells expressing informative markers, or in the case of peripheral blood, total number of cells expressing those markers per microliter), as well as a subjective description of the study and interpretation of the findings. Any reporting of values should be accompanied by reference ranges established by the laboratory; either percentages of different lymphocyte subpopulations for samples of bone marrow and lymph nodes, or absolute numbers of each cell type for peripheral blood. A small number of reference ranges for blood, bone marrow and lymph nodes have been published.^{17,42,53}

Reactive Versus Neoplastic

Two criteria can be used to establish that a population of cells are malignant: (1) The presence of cells with an

aberrant immunophenotype, and (2) in cases where the cells are lymphocytes, an expansion of lymphocytes with a homogeneous phenotype (for example all of the lymphocytes are B cells). We have suggested the following criteria for establishing that a population of lymphocytes in canine peripheral blood is malignant when such a determination by cytology is ambiguous,⁵⁴ but similar guidelines have not yet been suggested for distinguishing reactive from neoplastic processes in lymph nodes:

1. A lymphocyte count above the reference range for the submitting laboratory or clinic, and one of the following:
 - 2a. 80% of the lymphocytes have a single phenotype), or
 - 2b. 60% of the lymphocytes have a single phenotype and a positive clonality assay (PARR assay or PCR for antigen receptor rearrangements), or
 - 2c. Presence of lymphocytes with an aberrant phenotype for peripheral blood.

One well-established exception to the notion that homogeneous expansion of lymphocytes is diagnostic for malignancy is the observation that *Ehrlichia canis* infection also induces the exclusive expansion of CD8 T cells (large granular lymphocytes (LGL)) in a minority of chronic infections.^{19,48} Therefore if *E. canis* infection is a consideration, serology should be used to rule out this disease in cases of CD8 T cell expansion up to 30,000 cells/ μ L.

Cells can be considered to have an aberrant phenotype if the particular constellation of antigens they express is not found in normal or reactive processes, or not found in significant numbers. Aberrant phenotypes for dogs reported in the veterinary literature include: T cell leukemias/lymphomas that have lost expression of CD45,⁵⁴ co-expression of CD4 and CD8 on the same T cells, which indicates the presence of thymoma when found in a mediastinal mass aspirate,²⁴ or lymphoma/leukemia when found in peripheral lymph node aspirates or peripheral blood,^{46,53} expression of CD3 and/or CD5, but not CD4 or CD8,^{46,54} the expression of CD79a but not CD21 in B cell lymphomas,⁵³ and the expression of CD34 on acute leukemias.^{36,47,54} Therefore, the presence of these cells can be considered strong evidence supporting the diagnosis of lymphoma or leukemia.

There is significantly less information available for feline leukemia/lymphoma. Large granular lymphocytic leukemias in cats represent blood involvement secondary to intestinal lymphoma.³⁰ These tumors were most often CD8 T cell and CD3e positive, with variable levels of CD5 expression. Anecdotal experience from our laboratory and others⁵⁶ suggests that the majority of feline chronic lymphocytic leukemias are CD4+. We have also found that a subset of feline CLL express CD5 but not CD4, or only low levels of CD4. Clearly, however, a greater array of reagents would be very useful in the diagnosis of feline leukemia and lymphoma.

Persistent mature neutrophilia, monocytosis or eosinophilia often suggests a diagnosis of chronic myelog-

enous, myelomonocytic or eosinophilic leukemia. None of these conditions can be diagnosed by flow cytometry with currently available antibodies, because the normal cellular counterparts of these leukemias have the same immunophenotype as the leukemic cells.

Lineage Determination

Lymphocytic or Myeloid Origin Leukemia?

Leukemias of mature leukocytes (lymphocytes, neutrophils, monocytes) do not pose a dilemma for lineage determination because they each have a characteristic cytologic appearance. Leukemias of immature cells, however, can be more of a challenge. Four separate studies used failure to express lymphoid surface markers (CD3, CD8, CD21) or cytoplasmic CD3e or CD79a, in addition to the presence of characteristic morphology, to make the diagnosis of AML in dogs.^{39,43,46,47} The expression of cell surface CD14, CD4, CD11b, CD11c and neutrophil surface antibody (NSA), and/or intracellular expression of myeloperoxidase (MPO) and MAC387, have also been used, together with the above criteria, to establish a myeloid lineage.^{43,46,47} Conversely, staining with one of the lymphoid lineage markers described above, or positive staining for cytoplasmic CD3 or CD79a is considered diagnostic for lymphoid leukemia.

Acute or Chronic Leukemia?

Leukemic processes with >20% or 30% blasts in the peripheral blood or bone marrow are considered acute leukemias (Chapter 65).²⁰ More recently, CD34 expression has been recognized as diagnostic for acute leukemia^{43,47,54,56} but the absence of CD34 expression does not rule out acute leukemia. Villiers et al. described 8 cases of acute myeloid leukemia, and provided a thorough description of their cytologic and criteria for making this diagnosis (>30% blasts in the peripheral blood or bone marrow, characteristic morphology). Two of these cases were CD34 negative.⁴⁷ Vernau et al. suggested that acute LGL leukemia is also CD34 negative, but the criteria used for the diagnosis in these cases were not given in detail.⁴⁶ Presently, then, expression of CD34 can be considered diagnostic for acute leukemia, but absence of CD34 does not rule out AML. More detailed studies of CD34 expression on suspected ALL is warranted.

Leukemia or Lymphoma?

This is a question frequently posed by clinicians in the face of peripheral lymphocytosis, and the answer can impact treatment decisions. At present there is no way to make this distinction by immunophenotyping alone, but immunophenotyping can contribute to this decision. In our study of 99 cases of lymphocytosis, all cases fell into one of three main groups; CD34+ and negative for other surface markers (intracytoplasmic staining was not performed), B cells expressing CD21, and T

cells that were CD4–CD8+CD3+ or CD4–CD8–CD3+. CD4+ lymphocytosis is extremely rare. CD34-expressing tumors behaved clinically like acute leukemias, with their attendant poor prognosis. Lymphocytosis involving small cells with a mature B cell immunophenotype behave clinically like chronic lymphocytic leukemia.⁵⁴ Lymphocytosis involving T cells with a mature CD8+ phenotype and an initial lymphocyte count of less than 30,000 cells/μL also behaves clinically like CLL. Therefore, it seems reasonable to favor a diagnosis of chronic lymphocytic leukemia in these two situations. Cases involving large B cells or T cells and a high initial lymphocyte count may be more difficult to classify based on immunophenotype alone. These behave more aggressively, and using other criteria may be classified as acute leukemia or stage V lymphoma.

CLONALITY

Principles of Testing

Clonality testing refers to the process of determining if a population of cells is derived from a single parental clone, or if that population is heterogeneous. Clonality testing in lymphocytes is based upon the observation that during the course of an immune response a diverse array of lymphocytes are activated. Normal lymphocyte differentiation is dependent upon the rearrangement of genes encoding antigen receptors – Ig and TCR genes.^{5,13,21} During this process (Fig. 143.1) nucleotides are trimmed or added between genes as they recombine, resulting in significant length and sequence heterogeneity within the complementarity determining region 3 (CDR3). The end result of this differentiation is a diverse population of lymphocytes with virtually limitless antigen specificity and a large variety of CDR3 sequences and lengths.

Lymphocytes which are the product of division by a single parental cell will have CDR3 regions of the same length and sequence. Uniform CDR3 length can be established by amplifying DNA from the cells of interest with PCR primers directed at conserved regions of the V and J gene segments of Ig and TCR genes (schematically depicted in Figure 143.1). Amplified DNA is then separated by size using one of a variety of methods, including polyacrylamide gel electrophoresis and GeneScan analysis. The presence of a dominant single sized product indicates the presence of a group of lymphocytes that share an identically sized CDR3 region, i.e. that are clonally expanded. The presence of many sized products suggests a polyclonal population of lymphocytes.

In human medicine, determination of clonality is the test of choice if other diagnostics are ambiguous.³⁷ Clonality testing is now also available for dogs on a routine basis. We have named this assay the PARR assay (PCR for antigen receptor rearrangements) to distinguish it from other methods of clonality testing, but it is important to note that the term PARR does not appear in literature describing the assay in human

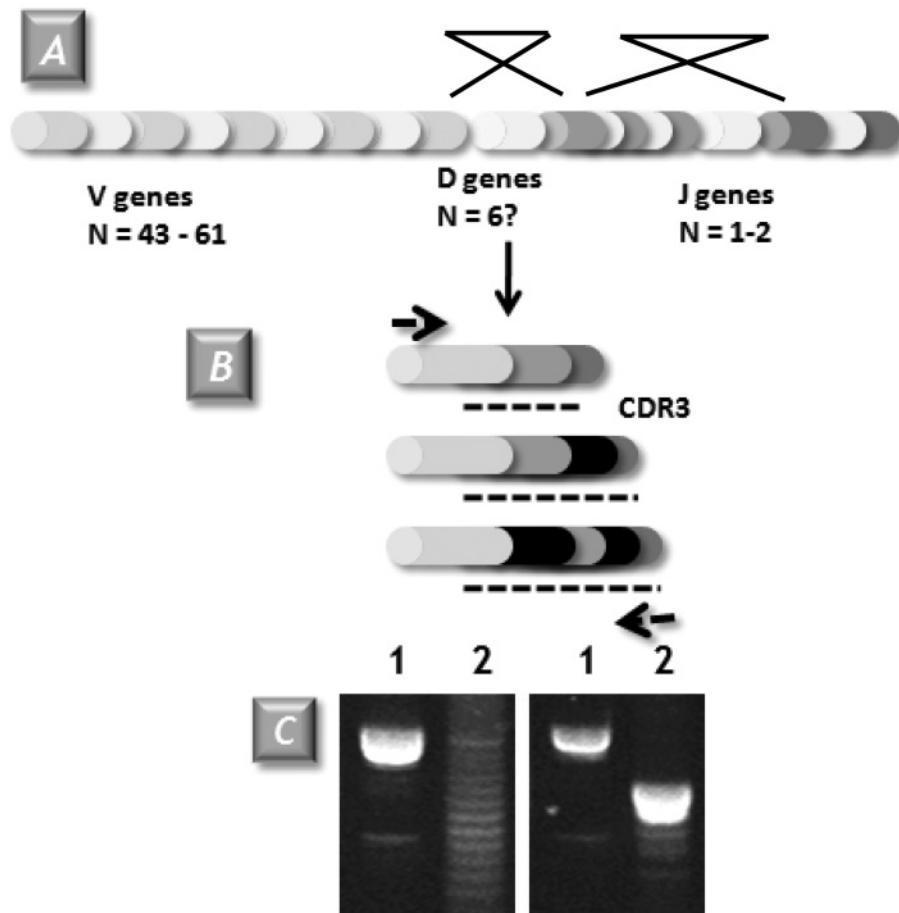


FIGURE 143.1 Immunoglobulin gene rearrangement results in CDR3 regions of variable sizes. (A) DNA (white) between V region genes (light gray) and D region genes (medium gray), and between D region genes and J region genes (dark gray) loops out to bring V, D and J gene segments together. (B) The CDR3 region (dashed line) is the result of recombination between V, D and J region gene segments. The length of this region varies between B cells because nucleotides are added (black segments), and end to end joining between V, D and J genes is imprecise. Polymerase chain reaction primers placed outside of CDR3 (arrows) in the conserved area of the gene will amplify intervening DNA resulting in different sized products from different B cells. (C) Polyacrylamide gel electrophoresis of PCR products from a dog with reactive lymphoid hyperplasia (left) and from a dog with lymphoma (right). The first lane of each panel shows amplification with primers specific for a control gene (any non-antigen receptor gene) as a positive control for DNA. The second lane shows DNA amplified with immunoglobulin primers. DNA from the dog with lymphoid hyperplasia yielded multiple faint PCR products. DNA from the dog with lymphoma yielded a single sized product. Similar principles apply to TCR gamma genes, except that these genes lack D regions.

patients. The first large scale study of this technique was reported by Burnett et al,⁸ following earlier studies that demonstrated the presence of clonally rearranged TCR genes in canine malignancy.^{15,18,46} A number of laboratories have reported versions of the assay.^{6,8,22,38,46,57,58}

Each laboratory performing this test uses slightly different primer sequences,^{6,8,22,38,46,57,58} but all laboratories use primers which anneal in the framework 3 region of Ig and TCR V genes, and to the 3' end of Ig and TCR J genes. The TCR gamma gene is used for this assay despite the fact that the majority of T cells express the α/β T cell receptor protein, because in the thymus, the TCR gamma gene rearranges before the TCR beta gene. If this rearrangement is not productive (which is true the majority of the time), the T cell will go on to rearrange TCR beta, but the rearranged TCR gamma gene

remains²⁹ and can still be used as a marker of clonality. Early studies in human patients found this assay to be more sensitive than detection of TCR beta rearrangement,^{14,40,41} and therefore TCR gamma was also used in the development of the assay for canine and feline patients. Sequencing of feline TCR gamma⁴⁹ and Ig genes⁵² has been reported and used clinically⁹ but these assays are less well developed.

Obtaining and Shipping Samples

Virtually any source of cells can be used, including cytology slides regardless of whether they are stained or not. In addition to stained slides, aspirates from masses or nodes can be squirted directly into an EDTA tube with the addition of other liquid. The appropriate sample to submit for clonality testing is the tissue con-

taining the suspicious cells: testing of peripheral blood in cases where there is no obvious blood involvement is significantly less sensitive than testing the suspicious lymph node,²⁵ mass or cavity fluid.

The only sample type not used by our laboratory is DNA derived from formalin fixed, paraffin embedded tissues. The process of formalin fixation substantially decreases the quality of the DNA, and different sensitivity and specificity parameters must be developed for this sample source. Useful information can come from the analysis of formalin fixed samples, however, and this testing is performed in some laboratories.⁴⁴

Quality Control

As with all PCR-based assays, negative controls that include all elements of the PCR reaction except the DNA to be tested are essential to demonstrate there is no contamination. In addition, it is also important to establish that there is sufficient DNA in a particular clinical sample for detection in order to make both negative and positive results meaningful. While insufficient DNA may result in false negative results, it can also lead to false positives which are sometimes termed "pseudoclonal." Although it may seem paradoxical, in a sample of low cellularity or poor quality DNA (such as formalin fixed samples), the intact DNA that is present may be from only a small number of cells. In these cases, amplification will seem to yield a clonal result because few Ig or TCR genes are present to bind the primers.¹⁶

There are at least two ways to control for this phenomenon. Some groups carry out all analyses in duplicate⁴⁴ because pseudoclonal PCR products are often not repeatable. Our laboratory prefers to amplify a non-antigen receptor gene as a positive control for DNA (Fig. 143.1).⁸ This indicates there is sufficient DNA present, and therefore validates negative results obtained with Ig and TCR primers.

Sensitivity and Specificity

When first developed, clonality testing in people^{1,2,4,14} had sensitivity similar to what we currently achieve in dogs: 75–80%. The addition of detection of light chain gene rearrangement has increased the sensitivity to close to 100%.⁴⁵ Specificity values for the assay in human patients are harder to find. In one, 80 cases of atypical or benign lymphoid hyperplasia were tested for clonality, and in all cases where a clonal population of T or B cells was detected ($n = 5$), the patients went on to develop malignancy within 2 years.³³ This finding suggests that clonality testing is able to detect malignancy before conventional diagnostics.

In veterinary medicine, clonality testing almost certainly differs in sensitivity and specificity between laboratories, and these values need to be established by each institution offering the assay. Our laboratory detects approximately 75% of histologically or cytologically confirmed lymphomas, when the assay is carried out on the cells in question (based on testing of approximately

800 cases). Lack of a clonally rearranged antigen receptor on a confirmed case of lymphoma or leukemia can have several explanations: (1) the genes involved in the antigen receptor rearrangement in this tumor are not detectable by our primers (details of V and J region TCR and Ig genes in dogs have been previously described^{11,38,57}); or (2) there is no antigen receptor rearrangement because the tumor is NK in origin; or (3) the tumor is derived from an early lymphocyte precursor. These types of tumors may be characterized by flow cytometry. PARR negative malignancies do not appear to have a different prognosis than PARR positive malignancies.²⁵

In order to determine the specificity of a positive finding, we tested a series of 72 cases with clinical conditions other than lymphoid malignancy and found a false positive rate of 8%.⁷ The most common reason for detecting a clonal T cell population in a dog without neoplasia is *E. canis* infection, a phenomenon that has also been reported in the literature.^{8,46} Each case of *E. canis* was diagnosed by serology, responded to therapy and was healthy at a minimum of 8 months follow-up. Rarely, cases of Lyme disease, bartonellosis and Rocky Mountain spotted fever have been associated with clonal B cell expansion, but this finding is unusual (A.C. Avery, unpublished results). Leukemias with a suspected or confirmed myeloid origin were excluded from this case series because in people²³ and in dogs⁸ acute myelogenous leukemias (AML) can have aberrantly rearranged lymphocyte antigen receptor genes.

Interpretation of Results

Possible results of a clonality assay include: no amplification of DNA using TCR or Ig primers, amplification of one or two products (clonal amplification), amplification of three or more products that are not regularly spaced in size (oligoclonal), amplification of a range of PCR products that are evenly distributed with regard to size (polyclonal). Samples from non-lymphoid tissues tend to yield the first kind of result (no PCR products except the positive control). This is because the distance between two primers annealing to un-rearranged Ig or TCR genes is too long to successfully amplify PCR product. Samples from normal or reactive lymphoid tissue tend to yield polyclonal PCR products.

One or two PCR products indicate the presence of one or two clonal populations of lymphocytes: a single tumor can have rearrangements of antigen receptor genes on both chromosomes. Oligoclonal amplification is more difficult to interpret. In our experience (A.C. Avery, unpublished observations), T cell lymphomas unambiguously diagnosed by cytology or histology often have multiple clonal PCR products. This observation has also been reported in certain kinds of human T cell lymphomas.³ The explanation for these findings is not clear, but includes the possibility of ongoing gene rearrangement during tumor progression, or a process that involves malignant transformation of more than one T cell precursor simultaneously. Sequencing of these clonal PCR products may help clarify this issue.

CLONALITY TESTING OR IMMUNOPHENOTYPING IN LYMPHOID LEUKEMIA AND LYMPHOMA – WHICH TO CHOOSE?

Each laboratory that provides these diagnostic tests may have different recommendations, but in general, because immunophenotyping provides substantially more information than clonality testing, we recommend clonality testing only in cases where the cytologic or histologic diagnosis is ambiguous. When there is a definitive or highly probable diagnosis of lymphoma or leukemia, immunophenotyping by flow cytometry should virtually always be able to confirm neoplasia by detecting cells with an aberrant phenotype or a homogeneous expansion of lymphocytes. In addition, any peripheral blood samples with an elevated lymphocyte count should be assessed by flow cytometry, because this assay will provide both a diagnosis and in many cases a prognosis for the canine patient.⁵⁴

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Cytochemical Staining

ROSE E. RASKIN

General Principles of Cytochemistry
 General Considerations for Staining Neoplastic
 Hematopoietic Cells
 Reactions of Normal Hematopoietic Cells
 Neutrophils/Heterophils
 Eosinophils
 Basophils
 Monocytes/Azurophils
 Lymphocytes
 Platelets and Megakaryocytes
 Erythroid Cells
 Mast Cells
 Cytochemical Staining Principles and Application
 Myeloperoxidase

Sudan Black B
 Chloroacetate Esterase
 Leukocyte Alkaline Phosphatase
 Periodic Acid-Schiff
 Nonspecific Esterases
 Acid Phosphatase
 Beta Glucuronidase
 Miscellaneous Stains
 Toluidine blue
 Omega exonuclease
 Synacril black AN
 Acetylcholinesterase
 Luna
 Summary

Acronyms and Abbreviations

ACH, acetylcholinesterase; ACP, acid phosphatase; ALL, acute lymphoblastic leukemia; ANAE, alpha naphthyl acetate esterase; ANBE, alpha naphthyl butyrate esterase; β G, β -glucuronidase; CAE, chloroacetate esterase; LAP, leukocyte alkaline phosphatase; NK, natural killer; NSE, nonspecific esterase; OEN, omega-exonuclease; PAS, periodic acid-Schiff; PER, peroxidase; SBB, Sudan black B; TB, toluidine blue.

GENERAL PRINCIPLES OF CYTOCHEMISTRY

Cytochemistry is the use of special stains in the microscopic examination of cellular constituents such as lipids, carbohydrates, and enzymes.⁵⁷ These stains may detect differences between normal cell types and identify the lineage of poorly differentiated blast cells (see Section V on Hematologic Neoplasia). Cytochemistry is ideal as a diagnostic technique because it involves minimal cost and is readily performed at diagnostic referral centers. This technique may be used with hemolymphatic tissues (blood, bone marrow, lymph nodes, spleen, and thymus) or with liver. These specimens may include fresh imprints or aspirates, frozen tissues,⁷⁹ or plastic-embedded tissues.⁸ In contrast, paraffin-embedded tissues may not be stained for nonspecific esterase (NSE) activity because the enzyme is unstable during tissue processing. In addition to light microscopic preparations, cytochemical staining also has been applied to electron microscopic specimens.^{6,49}

To ensure proper sample handling, the clinician should contact the laboratory performing the cytochemical staining before submitting materials. Multiple unfixed slides usually are preferred so a panel of stains can be performed. During cytochemical staining, appropriate control slides are used as positive and negative stain markers to assure quality control for the procedure(s). Although cytochemistry is applicable to the cells of various species, it is noteworthy that incubation times, staining patterns, and the presence of substrates may differ, even among the various avian, reptile, and fish species.

GENERAL CONSIDERATIONS FOR STAINING NEOPLASTIC HEMATOPOIETIC CELLS

The first step in the identification and classification of hematopoietic neoplasia is a morphologic characterization of the poorly differentiated blast cell population

and accompanying cells. This may be followed by special tests to better define the cell of origin. Poorly differentiated cells are indistinguishable by morphology alone but may be better identified by their cytochemical characteristics. This is critical for acute leukemias and especially useful in domestic animals like the dog, cat, and horse. The use of cytochemical staining can improve the diagnosis and sometimes change the initial morphologic interpretation.^{27,28,35}

The general premise of cytochemistry for hematopoietic malignancies is that the neoplastic cells contain the same enzymes or cellular products as are found in cells from healthy individuals. However, occasional irregularities may occur related to the altered development of neoplastic or dysplastic cells. Cytochemical staining is best interpreted when a panel rather than one or two stains are performed, since discrepancies may occur with neoplastic cells.

In general, lymphoid cells do not stain with many cytochemical stains. However, negative staining does not automatically indicate a lymphoid neoplasm. Rather it may represent an undifferentiated myeloid malignancy. Stain intensity is often very weak or absent in the most undifferentiated cells because they contain few of the necessary enzymes or cellular constituents found within lysosomal granules, cytoplasm, or the plasma membrane. On the other hand, some enzymes such as myeloperoxidase found in the primary granules of granulocytes are present to a greater extent in the earlier stages. In cytochemistry, it is best to interpret the results of positive staining rather than the lack of staining. Positive staining is meaningful, whereas negative staining leads to uncertainty.

In addition to stain intensity, the pattern of staining may be diagnostic. T-lymphocytes bearing focal lysosomal granules often produce a focal or discrete localization of NSE enzyme activity compared with the diffuse or granular nature found in some nonlymphoid

cells. Lymphoid cells are best classified using immunologic markers, but lineage- and species-specific monoclonal antibodies may be limited in availability or by cost. Therefore, limitations using immunomarkers supports continued use of cytochemical staining techniques for T-cell identification.⁸³

In the interpretation of staining data, additional factors are considered. One factor is the species involved. Reactions seen within one species cannot be extended readily to other species. For example, feline eosinophils normally do not stain with either peroxidase (PER) or Sudan black B (SBB), whereas these stains can be used in the eosinophils of other species.⁴⁵ Another factor involves the extent of staining within the sample. The percentage of stained blast cells in the bone marrow should be sufficient to establish a positive reaction with a given stain. Generally, this is greater than 3%. In the case of PER or SBB, greater than 3% of the blast cells present should be positive to identify human myeloblastic leukemia (without maturation).⁹ It is best to count a minimum of 200 bone marrow cells when calculating this figure to avoid an over- or underestimation. A simplified classification of acute leukemias using cytochemical staining is shown in Fig. 144.1.

REACTIONS OF NORMAL HEMATOPOIETIC CELLS

A summary of normal cell reactions for selected stains in several animal species is provided in Table 144.1.

Neutrophils/Heterophils

Primary or azurophilic lysosomal granules contain myeloperoxidase. Peroxidase (PER) activity is readily demonstrated from the promyelocyte stage through mature segmented forms (Figs. 144.2A, B). In electron

FIGURE 144.1 Simplified algorithm for the classification of acute leukemia from blast cells in blood or bone marrow using cytochemical stains.

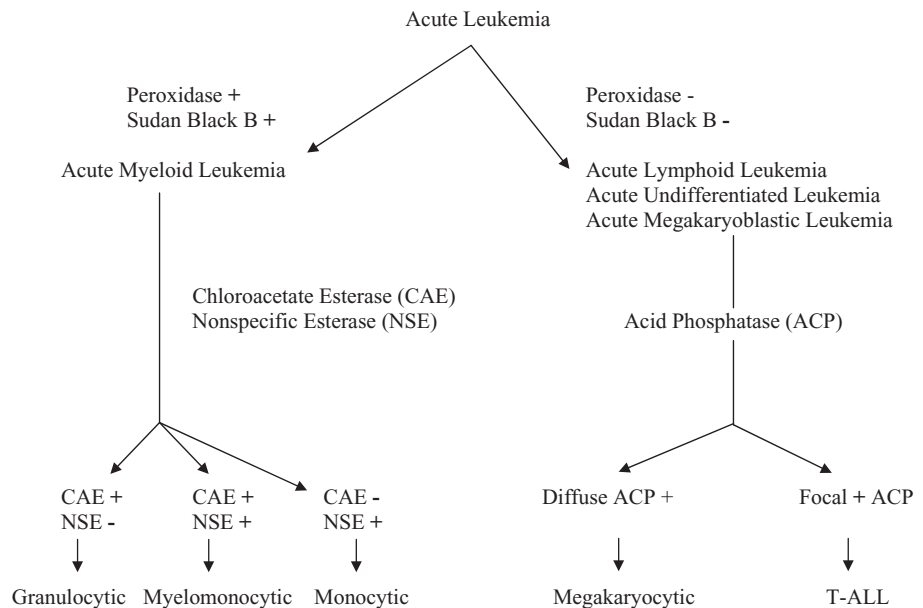


TABLE 144.1 Summary of Selected Cytochemical Staining Reactions for Normal Blood Cells in Various Domestic Animal Species^a

Species/Cell Type	PER	SBB	CAE	LAP	PAS	NSE	NSE/FL	ACP	ACP/T	BG
Bovine										
Neutrophil	pos	pos	pos	pos	pos	neg	—	pos/neg	—	—
Eosinophil	pos	pos	neg	pos	pos/neg	pos/neg	—	pos/neg	—	—
Basophil	neg	pos/neg	pos/neg	pos/neg	pos/neg	neg	neg	neg	neg	—
Monocyte	pos/neg	pos/neg	pos/neg	neg	pos/neg	pos/neg	pos/neg	pos/neg	—	—
Lymphocyte	neg	neg	neg	neg	pos/neg	pos/neg	pos	pos/neg	neg	—
Cat										
Neutrophil	pos	pos	pos	neg	pos	neg	neg	pos/neg	neg	neg
Eosinophil	neg	neg	neg	pos	pos/neg	neg	neg	pos	pos	neg
Basophil	neg	neg	pos	pos/neg	pos/neg	neg	neg	pos	neg	—
Monocyte	pos/neg	pos/neg	neg	neg	pos/neg	pos/neg	neg	pos/neg	neg	pos
Lymphocyte	neg	neg	neg	neg	neg	pos/neg	pos/neg	pos/neg	neg	pos/neg
Platelet/Megakaryocyte	neg	neg	pos/neg	neg	pos/neg	pos/neg	neg	pos/neg	neg	pos/neg
Dog										
Neutrophil	pos	pos	pos	neg	pos	neg	neg	pos/neg	neg	—
Eosinophil	pos	pos	neg	pos/neg	pos/neg	neg	neg	pos	pos	—
Basophil	neg	neg	pos	pos/neg	pos/neg	neg	neg	pos/neg	pos	—
Monocyte	pos/neg	pos/neg	neg	pos/neg	pos/neg	pos/neg	neg	pos	neg	—
Lymphocyte	neg	neg	pos/neg	pos/neg	pos/neg	pos/neg	pos	pos/neg	neg	—
Platelet/Megakaryocyte	neg	neg	pos/neg	neg	pos	pos	pos/neg	pos	neg	—
Horse										
Neutrophil	pos	pos	pos	pos	pos	pos/neg	—	pos/neg	—	—
Eosinophil	pos	pos	neg	pos/neg	pos/neg	pos/neg	—	pos/neg	—	—
Basophil	neg	neg	pos/neg	neg	pos	neg	—	neg	—	—
Monocyte	pos/neg	pos/neg	pos/neg	neg	pos/neg	pos/neg	—	pos/neg	—	—
Lymphocyte	neg	neg	neg	neg	pos/neg	pos/neg	—	pos/neg	—	—
Platelet/Megakaryocyte	neg	neg	—	neg	—	—	—	pos	—	—
Chicken										
Heterophil	neg	neg	neg	neg	pos/neg	neg	—	neg	—	—
Eosinophil	pos	pos	neg	neg	neg	neg	—	pos	—	—
Monocyte	pos/neg	pos/neg	—	neg	pos/neg	pos	—	pos	—	—
Lymphocyte	neg	neg	—	neg	neg	pos/neg	—	pos/neg	—	—
Thrombocyte	—	—	—	—	pos	—	—	—	—	—

^aPER, peroxidase; SBB, Sudan black B; CAE, chloroacetate esterase; LAP, leukocyte alkaline phosphatase; PAS, periodic acid-Schiff; NSE, nonspecific esterase; NSE/FL, nonspecific esterase fluoride; ACP, acid phosphatase, ACP/T, acid phosphatase with tartrate; BG, beta glucuronidase; neg, no reaction; pos, easily detectable; pos/neg, weak or focal or occasional reactivity; —, undetermined

microscopic preparations, PER activity also may be visualized in the nuclear membrane, rough endoplasmic reticulum, and Golgi apparatus of myeloblasts.⁴⁵ Peroxidase reactivity generally is absent in heterophils of rabbits, certain birds, and reptiles.^{3,53,101} Iguana heterophils, in contrast to many reptiles, have a significant positive PER reactivity.³⁷ Neutrophils with eosinophilic prominence, such as those of the manatee, possess myeloperoxidase and stain intensely.⁵³

Secondary or specific granules contain lipids, which stain positively with Sudan black B (SBB) (Fig. 144.3A). Sudan black B staining is found to a lesser extent within primary granules (Fig. 144.3B). Because both primary and secondary granules stain with SBB, all stages of neutrophil maturation are detected.

Primary and secondary neutrophilic granules also contain chloroacetate esterase (CAE) activity (Figs. 144.4A–C). The cytochemical stain technique detects enzymatic activity, producing moderate to strong reactions in all maturation stages of neutrophils (Fig. 144.5).

Leukocyte alkaline phosphatase (LAP) is found predominantly in the secondary granules of neutrophils of

most species including humans, horses, rabbits, and ruminants (Fig. 144.6). However, this enzyme is absent in more mature neutrophils of dogs and cats. Equine and caprine neutrophils stained for NSE activity may show variable reactivity; neutrophils from most other species are cytochemically nonreactive.^{45,95}

Acid phosphatase (ACP) activity is present in the primary granules of neutrophils. Enzymatic activity is higher in immature neutrophils than in bands or segmenters. Stain reactivity, however, is of modest intensity.

Eosinophils

The PER that is present in eosinophils differs both structurally and biochemically from that found in neutrophils and megakaryocytes. This enzyme is present in the matrix of large eosinophilic granules but not within the crystalloid core (Fig. 144.2A). Unlike eosinophils of most species, feline eosinophils do not stain with either PER or SBB techniques. However, PER and SBB reactivity may be observed in avian and reptilian

TABLE 144.2 Summary of Selected Cytochemical Staining Reactions for Normal Blood Cells in Various Exotic Animal Species^a

Species/Cell Type	PER	SBB	CAE	LAP	PAS	NSE	NSE/FL	ACP	ACP/T	BG
Elephant										
Heterophil	pos	—	pos	pos	—	neg	—	—	—	—
Eosinophil	pos	—	neg	neg	—	pos	—	—	—	—
Basophil	neg	—	pos/neg	—	—	—	—	—	—	—
Monocyte	pos	—	pos/neg	neg	—	neg	—	—	—	—
Lymphocyte	neg	—	pos/neg	neg	—	pos/neg	—	—	—	—
Thrombocyte	neg	—	neg	—	—	neg	—	—	—	—
Channel Catfish										
Neutrophil	pos	pos	neg	neg	pos	neg	—	pos	—	pos/neg
Basophil	neg	neg	neg	neg	pos	neg	—	neg	—	neg
Monocyte	neg	neg	neg	neg	neg	pos/neg	—	pos	—	neg
Lymphocyte	neg	pos/neg	neg	neg	neg	neg	—	pos/neg	—	neg
Thrombocyte	neg	neg	neg	neg	pos/neg	neg	—	neg	—	neg
Alligator										
Heterophil	neg	—	neg	pos	pos	pos	—	pos	—	—
Eosinophil	pos	—	neg	pos	pos	neg	—	neg	—	—
Basophil	neg	—	neg	neg	pos	neg	—	pos	—	—
Monocyte	neg	—	pos/neg	neg	pos	neg	—	neg	—	—
Lymphocyte	neg	—	pos	pos	pos	pos	—	pos	—	—
Thrombocyte	neg	—	neg	neg	pos	neg	—	neg	—	—
Iguana										
Heterophil	pos	pos	neg	neg	pos	pos	—	pos	—	—
Eosinophil	neg	neg	neg	neg	neg	neg	—	neg	—	—
Basophil	neg	neg	neg	neg	pos/neg	neg	—	neg	—	—
Monocyte/Azurophil	neg	neg	neg	neg	pos/neg	pos	—	pos	—	—
Lymphocyte	neg	neg	neg	neg	neg	neg	—	neg	—	—
Thrombocyte	neg	neg	neg	neg	pos	pos	—	neg	—	—
Green Sea Turtle										
Heterophil	neg	neg	neg	—	pos	pos	—	neg	—	—
Eosinophil	neg	neg	pos	—	pos/neg	neg	—	neg	—	—
Basophil	—	—	—	—	—	—	—	—	—	—
Monocyte	neg	neg	neg	—	pos	pos/neg	—	pos	—	—
Lymphocyte	neg	neg	neg	—	neg	neg	—	neg	—	—
Thrombocyte	neg	neg	neg	—	pos	pos	—	neg	—	—

^aPER, peroxidase; SBB, Sudan black B; CAE, chloroacetate esterase; LAP, leukocyte alkaline phosphatase; PAS, periodic acid-Schiff; NSE, nonspecific esterase; NSE/FL, nonspecific esterase fluoride; ACP, acid phosphatase; ACP/T, acid phosphatase with tartrate; BG, beta glucuronidase; neg, no reaction; pos, easily detectable; pos/neg, weak or focal or occasional reactivity; —, undetermined.

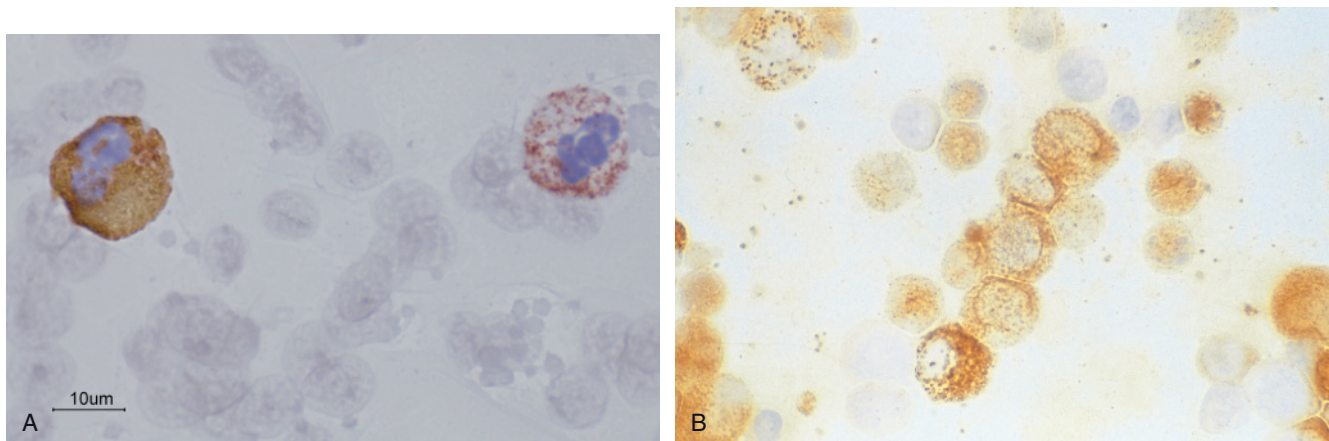


FIGURE 144.2 (A) Peroxidase (aminoethylcarbazole) reaction in elephant blood showing strong reaction in an eosinophil (left) and moderate reaction in a segmented heterophil (right). $\times 100$ objective. (B) Diffuse PER (benzidine) staining of immature cells in canine acute myelogenous leukemia. Bone marrow cytology. $\times 100$ objective.

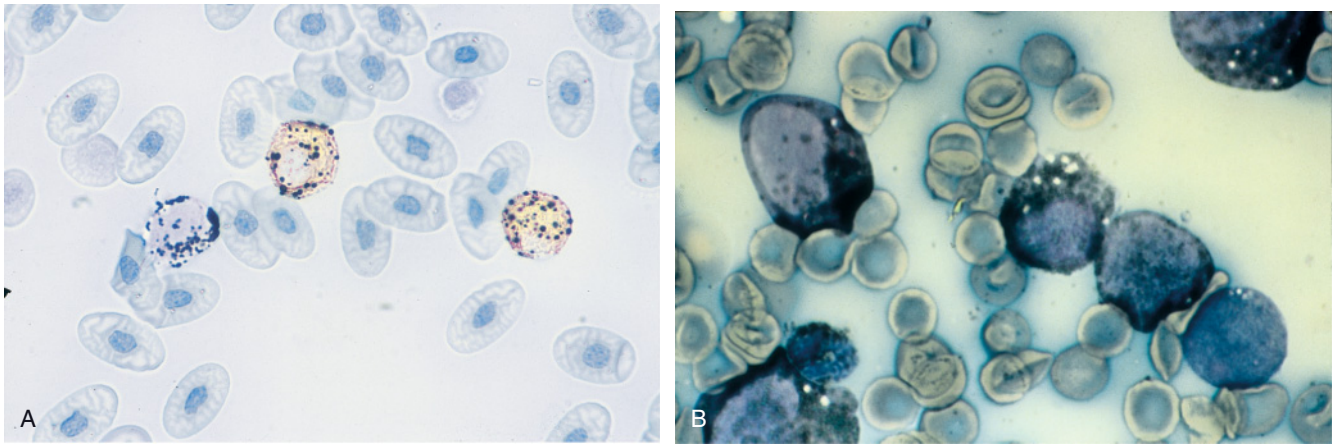


FIGURE 144.3 (A) SBB reaction in two heterophils and one azurophil from boa snake blood. $\times 100$ objective. (B) Diffuse SBB staining of blast cells in canine acute myelomonocytic leukemia. Bone marrow cytology. $\times 100$ objective.

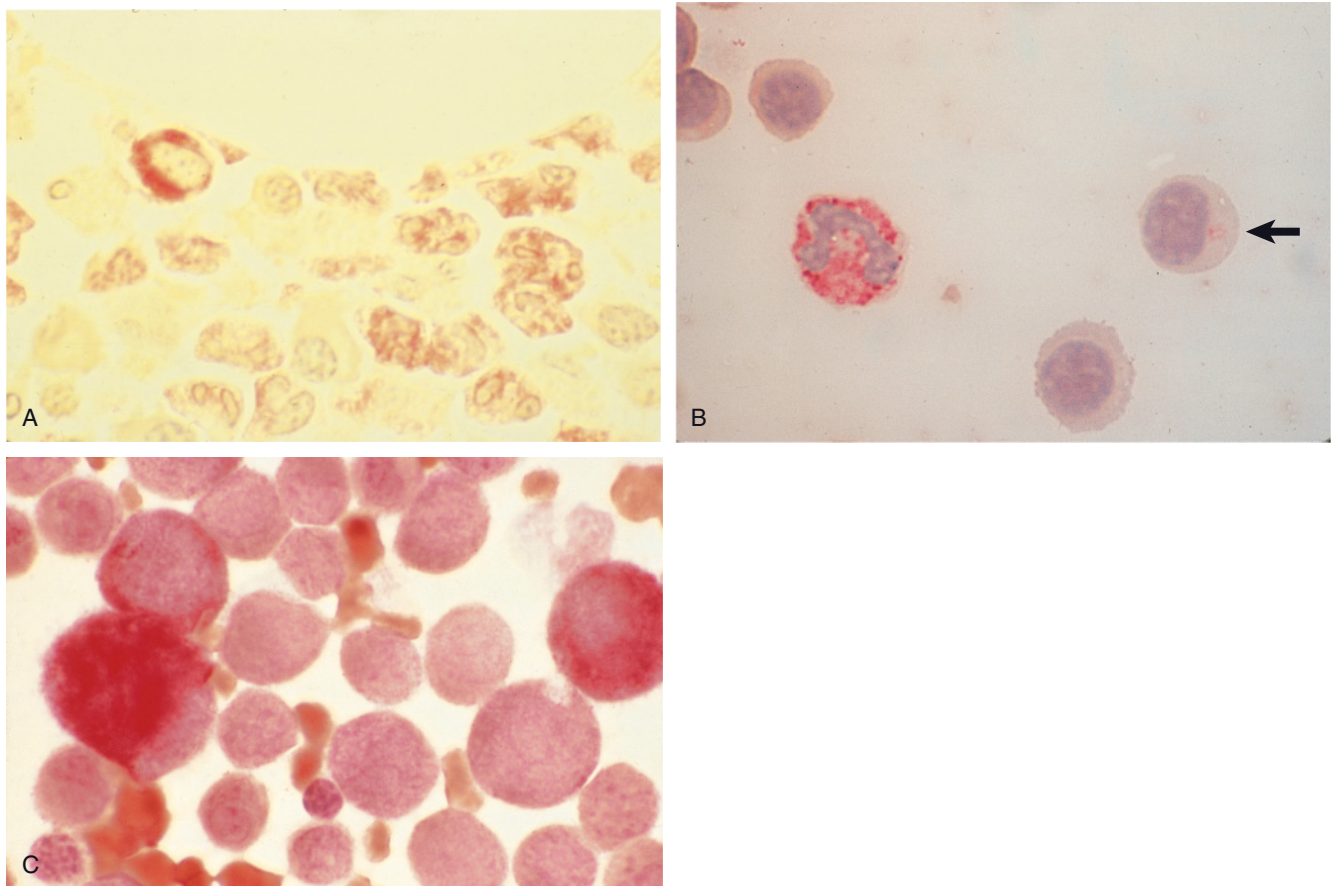


FIGURE 144.4 (A) Canine bone marrow with diffuse positive CAE reaction in granulocytic precursors. Plastic-embedded section. $\times 100$ objective. (B) Canine blood stained with CAE showing strong positive reaction in a neutrophil and focal reaction (arrow) in a lymphocyte. $\times 100$ objective. (C) Granulocytic blast cells stain CAE positive in feline acute myelomonocytic leukemia. Bone marrow cytology. $\times 100$ objective.

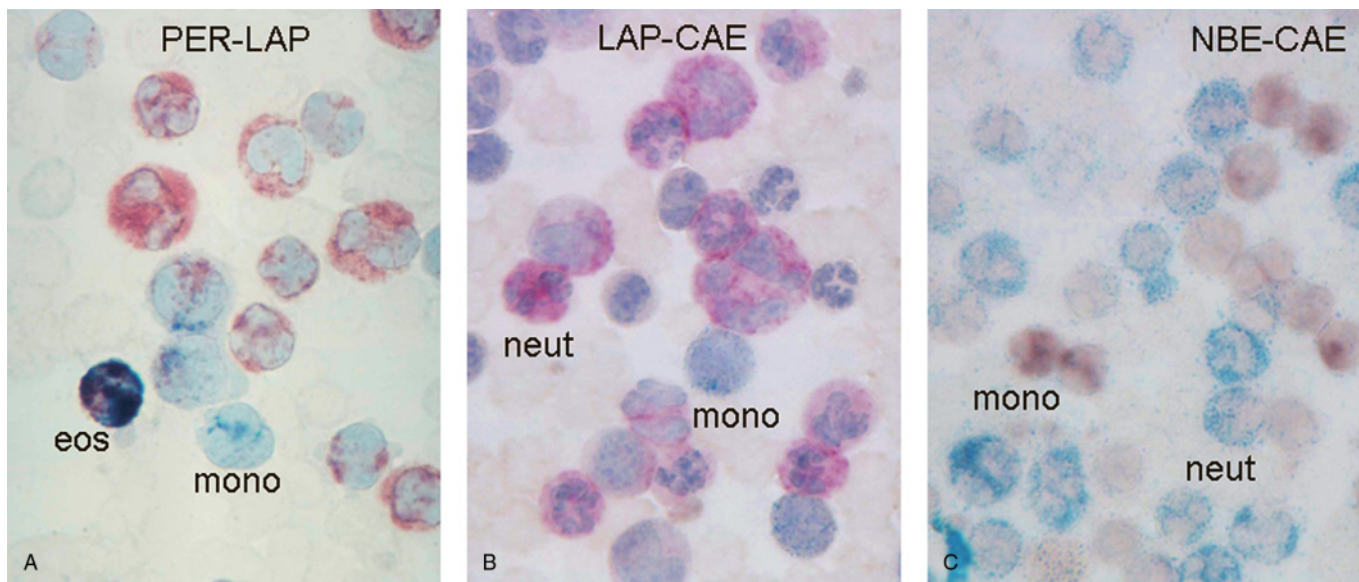


FIGURE 144.5 Double staining technique for canine bone marrow with chronic myelomonocytic leukemia. (A) Peroxidase activity (red) in neutrophils is coupled with leukocyte alkaline phosphatase (blue) that produces a strong reaction in between the eosinophil granules along with a mild reaction in monocytes; (B) mild leukocyte alkaline phosphatase activity (blue) in monocytes along with chloroacetate esterase activity (red) that reacts strongly with variable stages of neutrophils; (C) chloroacetate esterase activity (blue) in neutrophils is performed along with alpha naphthyl butyrate esterase (red-brown) indicating activity in monocytes. (Modified from cytochemical images provided by Dr. Julia Blue.)

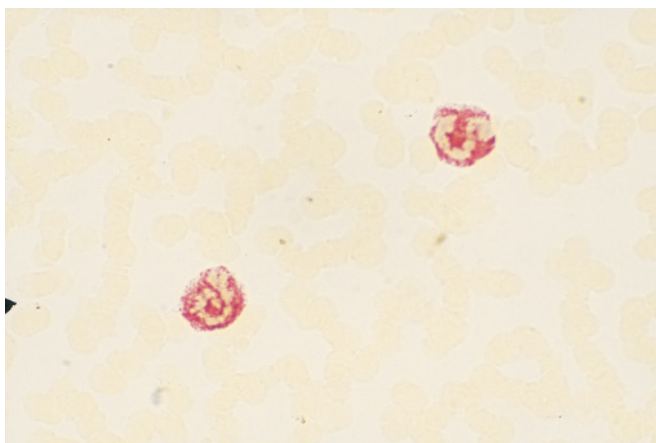


FIGURE 144.6 Equine blood with positive LAP in two neutrophils. $\times 100$ objective.

eosinophils.^{3,5,64} In general, eosinophils do not stain with CAE technique; however, reactivity has been observed in green turtle and koi eosinophils.^{94,101} Nonspecific esterase activity is absent in canine and feline eosinophils, but occasionally may be observed in equine and ruminant eosinophils.⁴⁵ Both alpha naphthyl butyrate esterase (ANBE) and alpha naphthyl acetate esterase (ANAE) activities are noted to be strong in elephant eosinophils (see Chapter 120). Eosinophils, especially those of the cat, stain strongly for ACP activity in the intergranular areas. Enzymatic activity is present in both canine and feline eosinophils and is tartrate-resistant.^{27,28} Leukocyte alkaline phosphatase activity is found intracellularly between the specific

granules of eosinophils (Fig. 144.5). The strongest reactivity occurs within the eosinophils of the horse. Specific eosinophil stains, such as Luna⁵⁹ and Luxol fast blue¹⁰⁶ also have been helpful in cell identification.

Basophils

Basophils are negative for SBB and PER reactivity. Feline and possum basophils may demonstrate occasional positive reactions with LAP activity.^{20,45} The CAE technique produces a moderately positive reaction in canine and feline basophils, whereas bovine and equine basophils are weakly positive for enzyme reactivity. Basophils of the dog, cat, horse, and cow are negative for NSE activity. Acid phosphatase activity occasionally may be observed in canine and feline basophils; enzymatic activity is tartrate-resistant.^{27,28}

Alcian blue stains positively for the sulfated mucosubstances in basophil granules of many species.⁷⁸ Omega-exonuclease (OEN) activity is present in basophils and mast cells of dogs and cats (Fig. 144.7A, B), but it is absent in both equine and bovine basophils.^{47,78} Toluidine blue (TB) staining may be metachromatic (purple) for basophil granules of domestic animals under acidic conditions, but this characteristic is not observed at neutral pH.⁵³ Toluidine blue staining also has been demonstrated in basophils from the alligator and desert tortoise.^{3,64}

Monocytes/Azuropils

Monocytes and azuropils of some species show diffuse, weakly positive reactivity for SBB and PER (Fig.

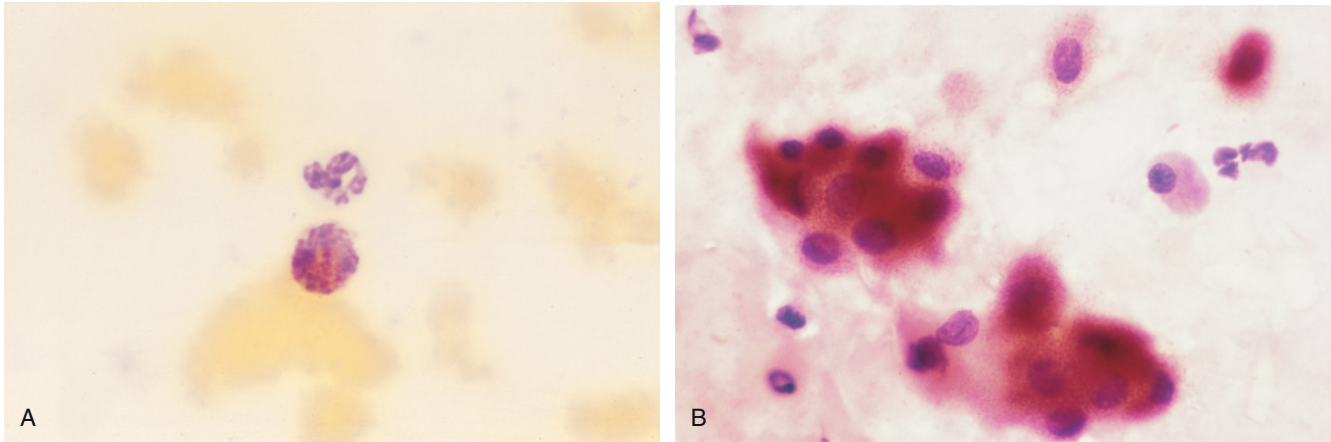


FIGURE 144.7 (A) Positive stain reaction with OEN in a feline basophil. Blood. $\times 100$ objective. (B) Strong cytoplasmic staining of cells from a canine mast cell tumor with OEN. Skin mass cytology. $\times 100$ objective.

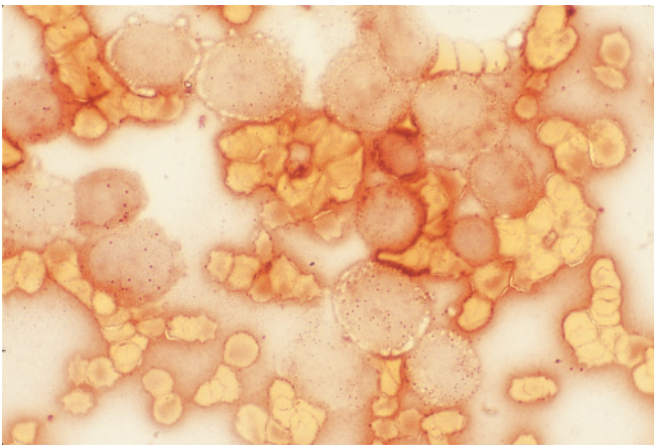


FIGURE 144.8 Diffuse ANAE staining of monocytoïd blast cells that were fluoride sensitive in equine acute monoblastic leukemia. Bone marrow cytology. $\times 100$ objective.

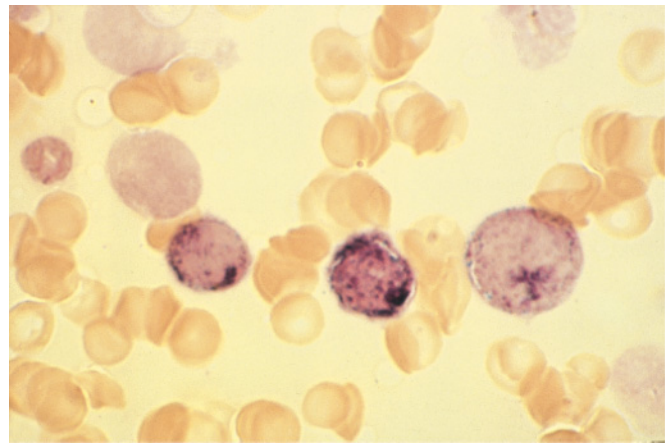


FIGURE 144.9 Diffuse LAP staining of blast cells in canine acute myelomonocytic leukemia. Bone marrow cytology. $\times 100$ objective.

144.5).^{2,4,11} Peroxidase activity may be more evident when copper nitrate is used to intensify the enzymatic reaction within the lysosomes. Generally, monocytes do not contain CAE activity; however, trace positive reactivity may be observed in bovine, equine, ovine, caprine monocytes⁴⁵ as well as the bilobed monocyte of elephants (see Chapter 120). Nonspecific esterase is present in cells of monocytic origin, resulting in diffuse, finely granular reactivity (Fig. 144.5) that is most intense in macrophages.^{42,103} Nonspecific esterase reactivity (Fig. 144.8) is inhibited with sodium fluoride due to the presence of the enzymes on the plasma membrane.^{13,25} Acid phosphatase reactivity is present in a diffuse manner, similar to that observed for NSE. Acid phosphatase reactivity is of moderate intensity in monocytes and increases with maturity into macrophages. Leukocyte alkaline phosphatase may be found in canine monocytes and monoblasts (Figs. 144.5 and 144.9) Monocytes in koi, an ornamental carp, are noted to have NBE as well as beta glucuronidase (β G) activities.⁹⁴

Lymphocytes

Traditionally, lymphocytes lack reactivity to many of the cytochemical techniques used to identify granulocytic and monocytic cells. Peroxidase activity is absent, although rare cases in human medicine exhibit PER expression as detected by molecular studies.⁴¹ Leukocyte alkaline phosphatase activity may be observed in a B-cell subtype associated with the mantle zone of lymph nodes in dogs.⁷⁹ Stain reactivity presents in a fine, diffusely granular pattern involving the plasma membrane. Chloroacetate esterase reactivity is rare in lymphocytes (Fig. 144.4B), but weak staining has been reported in bovine and equine lymphocytes⁴⁵ as well as in elephant lymphocytes (see Chapter 120). Occasional punctate staining has been associated with non-T-cell large granular lymphocytes in people.³⁹ Nonspecific esterase activity results in a focally discrete staining reaction within the intracellular organelles of normal and neoplastic lymphocytes from humans, dogs, cats,

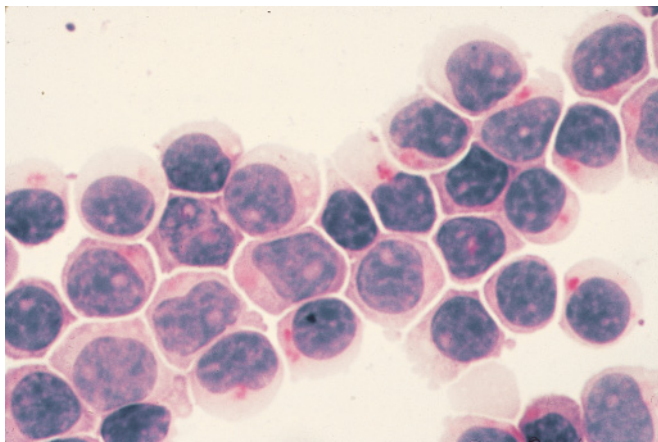


FIGURE 144.10 Focal stain reaction with ACP in canine lymphocytes having NK-cell activity. $\times 100$ objective.

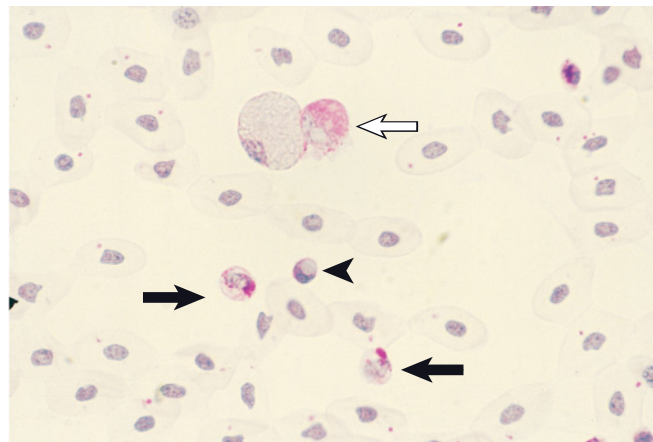


FIGURE 144.11 PAS reaction in boa snake blood cells showing strong reaction in thrombocytes (black arrows), moderate reaction in an azurophil (white arrow), trace reaction in a heterophil, and punctate reaction in erythrocytes. Note lymphocyte with inclusion body (arrowhead). $\times 100$ objective.

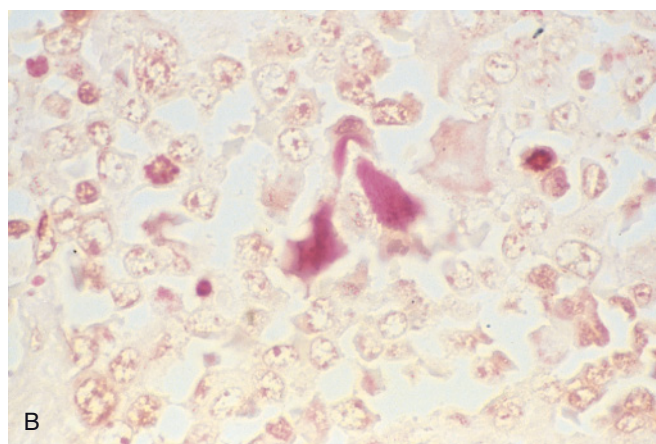
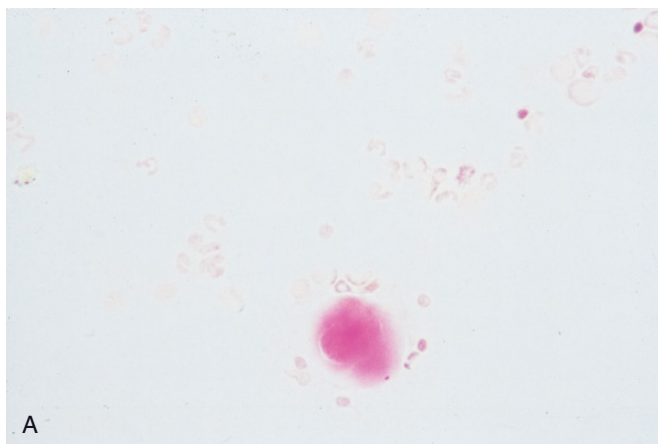


FIGURE 144.12 (A) Feline megakaryocyte with magenta reaction stained with Synacril black AN (Megacolor). Bone marrow. $\times 40$ objective. (B) Immature and mature megakaryocytic precursor cells stain positive with Synacril black AN (Megacolor) in canine acute megakaryoblastic leukemia. Bone marrow histopathology. $\times 100$ objective.

cattle, horses, sheep, elephants (see Chapter 120), and chickens. This focal staining has been associated with lymphocytes of a T-cell origin.^{6,25,27,28,39,75,98,102,104} Cells consistent with canine natural killer (NK) cell activity have weak, focal staining with the NSE and periodic acid-Schiff (PAS) techniques.⁵⁴ Weak PAS staining has been demonstrated in lymphocytes from cattle, dogs, horses, and sheep.⁴⁵ Positive reactivity for ACP has been observed in canine NK cells with and without tartrate incubation (Fig.144.10).⁵⁴

Platelets and Megakaryocytes

Platelets contain a specific PER that is only apparent in ultrastructural preparations (not in light microscopic specimens). This specific PER is present within the endoplasmic reticulum and nuclear membrane of megakaryocytes and within the tubular system of the

platelets. Slight CAE reactivity in canine and feline megakaryocytes has been reported, similar to that observed in human cells.^{27,28} Periodic acid-Schiff staining is often apparent within platelets and megakaryocytes of many animal species, including thrombocytes of birds and reptiles (Fig. 144.11).^{64,88} Positive reactivity to LAP- or SBB-staining techniques has not been demonstrated within platelets. Megakaryocytes and platelets may exhibit reactivity for NSE; these reactions are mildly to totally inhibited with sodium fluoride.³⁶ Both megakaryocytes and platelets are strongly reactive for ACP activity. Synacril black AN, a textile dye,⁵¹ selectively stains acid mucopolysaccharides found in the megakaryocytic cytoplasm (Figs. 144.12A, B); metachromatic staining is observed. Acetylcholinesterase is another marker that primarily identifies megakaryocytes and platelets in dogs, cats, and rats.⁴⁸

Erythroid Cells

Reactivity of erythroid cells to routine cytochemical stains is observed infrequently. Examples include a diffuse blush of positive reactivity due to endogenous PER activity. Similar observations occur with SBB cytochemistry. Acid phosphatase, β G, PAS (Fig. 144.11), and ANBE techniques may result in diffuse granular staining of various stages of erythroid precursors.^{27,28,96}

Mast Cells

Canine and feline mast cells exhibit variable reactivity with the CAE, PAS, alcian blue, toluidine blue, and OEN (Fig. 144.7B).⁴⁷ Mast cells, like basophils, are negative for PER and SBB reactivity. Leukocyte alkaline phosphatase activity is not present in canine and feline mast cells, whereas ACP reactivity results only in weak staining. Nonspecific esterase reactivity may have variable intensity in mast cells from some species like the pig, dog, and cat.

CYTOCHEMICAL STAINING PRINCIPLES AND APPLICATION

A listing of selected cytochemical stains and the methodology used by the author can be found in the previous edition of this book.⁸⁰ Other methods and kits may be used for a similar result including double staining techniques (Fig. 144.5). The cytochemical principle and application for each of the techniques will be further described below and shown in Tables 144.2 and 144.3.

Myeloperoxidase

This cytochemical technique is used as a marker for myeloid cells, particularly neutrophils, eosinophils, and monocytes (Fig. 144.2A). The PER enzymes are sensitive to tissue processing. Therefore, enzymatic reactivity is demonstrable only in fresh tissues and not in paraffin-embedded tissues. Peroxidase reactivity diminishes over time, especially in samples older than 2 weeks of age. Peroxidase reactivity may be less intense if smears are over 3 days old.³⁵ The most sensitive method to detect PER activity involves benzidine compounds that are potentially carcinogenic.⁵⁰ Copper salts enhance the benzidine reaction producing a gray-black product rather than an orange-brown result. An acceptable alternative substrate to diaminobenzidine tetrahydrochloride is 3-amino-9-carbazole.⁴⁶

The blast cells within the bone marrow should stain positive with PER for the following subtypes of acute myeloid leukemia: myeloblastic leukemia (with or without maturation), myelomonocytic leukemia, and erythroleukemia. Rare positive staining in monoblastic leukemia (without maturation) is possible. The intensity of staining within mature neutrophils reflects the level of activity within myeloblasts. There is a deficiency of PER activity for myelogenous leukemia in human medicine but during remission following chem-

otherapy, the activity increases and can be better detected in the mature neutrophil. Therefore monitoring PER activity may provide prognostic information regarding the efficacy of treatment in people.

Lymphoid malignancies traditionally lack PER reactivity; however, occasional reports in human medicine have identified molecular expression of the enzyme.⁴¹ There are no known reports of PER expression in veterinary cases of acute undifferentiated leukemia, basophilic leukemia, megakaryoblastic leukemia, acute lymphoid leukemia, lymphoma, histiocytic sarcoma, or mast cell neoplasia. Peroxidase staining was helpful in the diagnosis of canine, feline, equine, and porcine cases of myeloblastic leukemia (Fig. 144.2B) and myelomonocytic leukemia (Fig. 144.5).^{14,19,23,34-36,49,81}

Sudan Black B

Lipids (including phospholipids, neutral fats, and sterols) within neutrophils/heterophils, eosinophils, and occasional monocytes/azurophils are identified (Fig. 144.3A) by a gray-black reaction using SBB dye.⁸⁵ This staining technique is as sensitive as the PER reaction. The mechanism of cellular staining may involve an enzymatic reaction because eosinophils from patients that have eosinophil PER deficiency do not stain after SBB application. The same may be true in cats because their eosinophils also fail to stain. The sudanophilic material within cells is stable with heat or storage.

The blast cells within the bone marrow should stain positively with SBB (Fig. 144.3B) in the following subtypes of acute myeloid leukemia: myeloblastic leukemia (with or without maturation), myelomonocytic leukemia, monocytic leukemia, and erythroleukemia. Lymphoid malignancies traditionally lack positive reactions with SBB. However, one case of canine acute lymphoblastic leukemia displayed slight sudanophilia.²⁷ One case of canine malignant histiocytosis (now termed disseminated histiocytic sarcoma) had positive SBB reactivity.²⁷ There are no known reports of SBB reactivity in veterinary cases of acute undifferentiated leukemia, basophilic leukemia, megakaryoblastic leukemia, or mast cell neoplasia. Sudan black B positive expression was helpful in the diagnosis of canine, feline, equine, and bovine cases of myeloblastic leukemia, eosinophilic leukemia, monocytic leukemia, myelomonocytic leukemia, and erythroleukemia.^{14,27,28,32,61,70,77,81,100}

Chloroacetate Esterase

Referred to as specific esterase, the CAE reaction is likely produced by a group of enzymes. This reaction is considered more specific for neutrophils (Fig. 144.4B) than PER or SBB reactivity, although the CAE technique is less sensitive. Chloroacetate esterase reactivity is strongest from the promyelocyte to the segmented stage; weaker reactivity is observed in myeloblasts (Fig. 144.4A). The CAE reaction also is specific for mast cells.

TABLE 144.3 Summary of Cytochemical Staining in 171 Selected Cases of Nonlymphoid Malignancies^a

Disease/Subtype/Species	No. Cases	PER	SBB	CAE	LAP	ANAE	ANBE	Fluoride	ACP	ACP/T	ACP/T	BG	PAS
Acute Undifferentiated													
Leukemia													
Cat	1	neg	neg	ND	ND	ND	pos/neg	ND	pos/neg	ND	ND	pos	ND
Myeloblastic													
Dog	5	pos	pos/neg	pos	pos	neg	neg	ND	pos	neg	neg	ND	neg
Cat	7	pos/neg	pos	pos	pos/neg	neg	neg	neg	pos	pos	pos	ND	pos
Horse	1	pos	pos	pos	neg	ND	neg	ND	pos	ND	ND	ND	pos
Swine	6	pos	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Eosinophilic													
Cat	2	neg	neg	pos/neg	pos	ND	pos/neg	ND	pos	pos	pos	ND	pos
Horse	1	ND	pos	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Basophilic													
Dog	2	neg	neg	pos/neg	neg	pos	neg	ND	pos	ND	ND	ND	pos/neg
Cat	1	neg	neg	pos	pos/neg	pos	ND	ND	pos	ND	ND	ND	ND
Myelomonocytic													
Dog	20	pos/neg	pos	pos	pos	pos	pos	neg	pos	neg	neg	ND	pos/neg
Cat	2	pos	pos	pos	pos/neg	pos	pos	neg	neg	ND	ND	ND	pos/neg
Horse	4	pos	pos	pos	neg	pos	pos	neg	ND	ND	ND	ND	ND
Bovine	1	neg	pos	pos	ND	pos	ND	ND	ND	ND	ND	ND	ND
Monoblastic													
Dog	10	pos/neg	pos/neg	neg	pos/neg	pos	ND	neg	ND	ND	ND	ND	pos/neg
Cat	3	neg	neg	neg	neg	pos	pos	neg	neg	neg	neg	ND	pos
Horse	1	ND	ND	pos/neg	pos/neg	pos	ND	ND	ND	ND	ND	ND	ND
Erythroleukemia													
Cat	4	neg	pos	pos	pos	neg	pos/neg	neg	pos	pos	pos	ND	pos/neg
Cat (erythroid predominance)	4	neg	neg	pos/neg	pos/neg	ND	neg	ND	pos/neg	pos/neg	pos/neg	ND	pos/neg
Dog (erythroid predominance)	1	neg	pos/neg	neg	pos/neg	ND	ND	ND	pos	pos	pos	ND	neg
Megakaryoblastic or ET													
Dog	10	neg	pos/neg	pos/neg	neg	pos	pos/neg	pos/neg	pos/neg	neg	neg	ND	pos
Cat	2	neg	ND	neg	neg	neg	neg	ND	neg	ND	ND	ND	pos
Histiocytic Sarcoma													
Dog	3	neg	pos/neg	pos	neg	pos	neg	ND	pos	pos/neg	pos/neg	ND	pos
Horse	1	ND	ND	ND	ND	ND	pos	ND	ND	ND	ND	ND	ND
Cat	2	ND	ND	ND	ND	ND	pos	neg	pos	ND	ND	ND	ND
Mast Cell Neoplasia													
Dog	39	neg	neg	pos	neg	ND	pos/neg	ND	pos/neg	ND	ND	ND	pos/neg
Cat MCT/Leukemia	2	neg	neg	pos	neg	ND	pos/neg	ND	pos/neg	pos/neg	pos/neg	ND	pos/neg
Goat	1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	pos
Cow	1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Pig	4	ND	ND	pos	ND	ND	pos	ND	pos	ND	ND	ND	pos/neg
Horse	30	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

^aPER, peroxidase; SBB, Sudan black B; CAE, chloroacetate esterase; LAP, leukocyte alkaline phosphatase; ANAE, alpha naphthyl acetate esterase; ANBE, alpha naphthyl butyrate esterase; ACP, acid phosphatase; ACP/T, acid phosphatase with tartrate; BG, beta glucuronidase; PAS, periodic acid-Schiff; ET, essential thrombocythemia; neg, no reaction; pos, easily detectable; pos/neg, weak or occasional reactivity.

This enzyme is found within the primary and secondary granules of neutrophils and within the specific granules of basophils. Although CAE is generally specific for neutrophils, other cell types have weak reactions. For example, mild positive reactions may occur within the monocytes of horses and ruminants. In addition, slight reactions within normal megakaryocytes of dogs and cats have been noted.^{27,28}

Enzymatic reactivity in the CAE procedure probably involves chymotrypsin and elastase. These enzymes are capable of hydrolyzing esters linked to the substrate naphthol AS-D chloroacetate that when, liberated, complex with a stable diazonium salt to produce an insoluble, colored product at the site of enzyme activity.⁶⁹ This esterase group can be distinguished biochemically from NSE on the basis of isoenzyme separation on gel electrophoresis.²⁶ This esterase group is considered specific related to its resistance to sodium fluoride to inhibit its stain reaction. Chloroacetate esterase staining may be performed together with a nonspecific esterase stain for monocytes to detect both neutrophil and monocyte populations simultaneously (Fig. 144.5).¹⁰³ The CAE cytochemical reaction is very stable and may be used in paraffin-embedded tissues.

Positive reactions with CAE occur in the following subtypes of acute and chronic myeloid leukemia: myeloblastic leukemia (with or without maturation), myelomonocytic leukemia (Figs. 144.4C and 144.5), and erythroleukemia. Chloroacetate esterase is less sensitive but more specific than PER in distinguishing granulocytic and monocytic precursors. In addition to its specificity for neutrophils, it is regarded as a marker for basophilic leukemia and mast cell tumors. Lymphoid malignancies generally lack positive reactions with CAE. However, one case each of canine and feline acute lymphoblastic leukemia (ALL) displayed a slight reaction.²⁸ This is similar to human medicine in which rare cases of positive staining have occurred in ALL. In addition CAE staining was noted in several feline lymphoma cases, many of which involved large granular lymphocytes.^{82,98} One case of feline eosinophilic leukemia was positive for CAE.²⁸ Occasional weak to moderate staining was noted in three cases of canine megakaryoblastic leukemia.^{21,67} One case of weak CAE staining occurred in a horse with monocytic leukemia.¹⁷ There are no known reports of CAE staining in canine or feline cases of monocytic leukemia. Chloroacetate esterase staining was helpful in the diagnosis of several canine, feline, bovine, equine, porcine, camelid, and saurian cases of myeloblastic leukemia, basophilic leukemia, myelogenous leukemia, myelomonocytic leukemia, malignant histiocytosis, mast cell tumor, and erythroleukemia.^{7,12,14,23,27,28,32,66,77,78,81,86,87,91,92,99,100}

Leukocyte Alkaline Phosphatase

Enzymes with LAP activity hydrolyze naphthol AS-BI phosphate at an alkaline pH, releasing naphthol to complex with a diazonium salt, forming an insoluble,

colored product near the site of enzyme activity.¹ This group of enzymes is present in granulocytes (Fig. 144.5), osteoblasts, histiocytes, vascular endothelial cells, and a subset of lymphocytes.^{26,71} In human neutrophils, LAP activity is present in a subset of granules associated with the plasma membrane. The LAP reactivity of neutrophils varies with the species. In people, metamyelocyte and earlier stages are devoid of reactivity, whereas more mature cells (such as segmenters) stain more intensely. Leukocyte alkaline phosphatase reactivity is normal to increased in leukocytosis or leukemoid reaction, but it is decreased in neoplastic neutrophils (e.g. chronic myelogenous leukemia). Leukocyte alkaline phosphatase reactivity, similar to that in people, occurs in rabbits, rats, guinea pigs, horses, cattle, sheep, goats, and monkeys.⁴⁵ In dogs and cats, the late stages of neutrophils are devoid of LAP activity, whereas enzymatic activity is present in myeloblast and promyelocyte stages. Therefore, the LAP technique has been helpful in identifying myelogenous leukemia. Leukocyte alkaline phosphatase activity is absent in human eosinophils. In contrast, LAP activity is strong in the equine eosinophils, moderate in bovine eosinophils, and mild to moderate in canine and feline eosinophils (Fig. 144.5). In each instance, enzymatic activity is observed in the cytoplasm between the specific granules. A subset of B lymphocytes, present in the mantle zone of lymph nodes, exhibits cellular membrane staining with LAP technique in people, dogs, rats, and mice.^{71,79} Leukocyte alkaline phosphatase reactivity, though present in a wide variety of cells and tissues, also is helpful for the identification of hemolymphatic cells from mammalian and certain reptilian species.

Because LAP may be found in neutrophil precursors, it has been suggested that the presence of enzyme activity is a specific marker for myelogenous origin of canine and feline leukemia.^{35,45} Specific subtypes of acute myeloid leukemia that display LAP (Fig. 144.9) activity include myeloblastic leukemia (with or without maturation), myelomonocytic leukemia, and erythroleukemia. Positive staining has been documented in cases of feline eosinophilic leukemia.^{28,89}

One feline case involving basophilic differentiation of an acute myeloid leukemia exhibited cytoplasmic LAP staining.¹² Lymphoid malignancies generally lack positive reactions with LAP. However, positive reactions in lymphoid malignancies have been noted.^{27,79,90,97} One case with weak LAP staining was documented in a horse that had monocytic leukemia.¹⁷ Leukocyte alkaline phosphatase staining has been observed in monoblasts from normal dogs and dogs that have monoblastic leukemia. (J. Blue, personal communication, 2005). There are no known reports of LAP staining in feline cases of monocytic leukemia or in canine and feline cases of histiocytic sarcoma (malignant histiocytosis), megakaryoblastic leukemia, and mast cell tumor. Leukocyte alkaline phosphatase staining was helpful in the diagnosis of several canine and feline cases of myeloblastic leukemia, myelomonocytic leukemia (Fig. 144.5), and erythroleukemia.^{23,27,28,32,34-36,62}

Periodic Acid-Schiff

In the PAS technique, periodic acid oxidizes carbohydrates to form aldehydes that react with Schiff's reagent to produce a dye product.⁵⁸ The bright red or magenta reaction product is focal, diffuse or granular. Depending on the species, PAS may be found within a wide variety of cells, including neutrophils, heterophils, eosinophils, basophils, monocytes, azurophils, lymphocytes, platelets, thrombocytes, and erythrocytes (Fig. 144.11). The materials stained include glycoproteins, mucoproteins, glycolipids, and high-molecular-weight carbohydrates. In blood cells, glycogen usually is the substance demonstrated; however, if the sample is predigested with diastase, glycogen staining is eliminated.

Mature forms of neutrophilic granulocytes and platelets or megakaryocytes stain the most intensely, with monocytes, eosinophils, and basophils producing a weak positive reaction. Myeloblasts and promyelocytes stain negatively and weakly positive with PAS, respectively.^{27,28} Lymphocytes usually do not stain positively, or if they do it is a very weak reaction, especially in plasmacytic forms.

In general, PAS staining in veterinary medicine is used to differentiate granulocytic or megakaryocytic precursors from lymphoid precursors. Specific subtypes of acute myeloid leukemia that may display PAS positive activity include myeloblastic leukemia, myelomonocytic leukemia, monocytic leukemia, erythroleukemia or erythrocytosis with erythroid predominance, and megakaryoblastic leukemia.^{21,27-29,36,38,77,81,93} Positive staining has been documented in canine histiocytic sarcoma (malignant histiocytosis).^{27,38} One case of feline eosinophilic leukemia displayed positive PAS reaction.²⁸ A weak stain reaction with PAS occurred in a case of basophilic leukemia.⁶⁶ Mast cell tumors in the dog, cat, goat, and pig have demonstrated positive PAS reaction.^{7,52,78,86} Infrequent or weak PAS staining has occurred with some feline and equine lymphoid malignancies.^{28,35,98}

Nonspecific Esterases

Nonspecific esterases compose a group of enzymes capable of hydrolyzing esters to liberate naphthol products that bind with a coupler dye. These esterases demonstrate a wide range of substrate specificity and stain pH environment for various blood cells; therefore, they are termed nonspecific. Many of the esterases, as determined by polyacrylamide gel electrophoresis, are cell specific. Monocytes, granulocytes, and a subset of lymphocytes exhibit unique patterns of reactivity. A red to pinkish brown, diffusely granular pattern is seen in monocytes (Fig. 144.5), macrophages stain more intensely, and a single focal or punctate reaction product is seen in T-lymphocytes. The NSE activity in monocytes is inhibited by the addition of sodium fluoride to the incubation medium, whereas T lymphocytes are resistant. Differentiated histiocytes or macrophages have weakened or abolished reactivity in the presence of sufficient sodium fluoride. Sensitivity to sodium

fluoride inhibition also has been observed in megakaryocytes, platelets, and plasma cells.^{25,36} The difference in inhibition relates to the location of the enzyme in these cells. For example, two NSE enzymes, alpha naphthyl acetate esterase (ANAE) and alpha naphthyl butyrate esterase (ANBE) have been identified mostly on the plasma membrane of monocytes but only within intracellular organelles of lymphocytes.^{13,24,39} The intracellular location of the enzyme appears to be protective. The activity of these enzymes is unstable, being sensitive to heat, fixatives, and storage. Fresh blood smears or tissue imprints are preferred to paraffin-embedded tissues that may not demonstrate the reaction.

Cell specificity for ANAE activity may be demonstrated by altering the pH of the medium, temperature, and length of incubation.^{13,57,72} Monocytes are best stained in an acidic medium, whereas both granulocytes and monocytes may show enzymatic activity when the same substrate is used in a neutral medium. Lymphocytes are best demonstrated only with acid conditions and prolonged incubation. Compared with ANBE, ANAE is more sensitive and less specific in identifying monocytes, macrophages, and T-lymphocytes. Therefore, sodium fluoride inhibition is necessary to differentiate the cell types. T lymphocytes have been identified by a focal staining pattern in humans, dogs, and chickens.^{6,102,104} The stain reaction may be weak in megakaryocytes, platelets, and plasma cells, all of which are sodium fluoride sensitive.³⁶

Similar to the ANAE technique, differential staining of cells for ANBE activity requires changing the pH of the incubation medium. Sodium fluoride inhibition often is not used with ANBE owing to its greater specificity for monocytes compared with ANAE.

Specific subtypes of acute myeloid leukemia that display positive NSE activity include myelomonocytic leukemia, monoblastic leukemia, and megakaryoblastic leukemia. Positive staining has been documented in cases of canine, feline, and equine histiocytic sarcoma (malignant histiocytosis).^{22,56,99} Weak stain reactions with ANAE occurred in feline and canine cases involving basophils.^{12,66} In addition, occasional positive ANBE reactions have been documented in canine, feline, and porcine mast cell tumors.^{7,28,78} One case of feline eosinophilic leukemia reported occasional positive staining with ANBE.²⁸ A cat with an acute undifferentiated leukemia, formerly termed reticuloendotheliosis, had diffuse ANBE staining in some of the leukemic cells.³⁰ One case of feline erythrocytosis was positive for ANBE.²⁸ Lymphoid malignancies may stain with NSE, giving a focal rather than diffuse appearance. Some examples of positive staining of lymphoid neoplasia may be found in dogs, cats, cattle, and sheep.^{16,27,28,31,35,75,79,82,90,97,98} There are no known reports of NSE staining in canine, feline, or equine cases of myeloblastic leukemia. In summary, ANAE has been helpful in diagnosing myelomonocytic leukemia, monocytic leukemia (Fig. 144.8), megakaryoblastic leukemia, blast cells of essential thrombocythemia, and histiocytic sarcoma (malignant histiocytosis), whereas ANBE has been helpful in diagnosing myelomonocytic leukemia,

monocytic leukemia, and histiocytic sarcoma (malignant histiocytosis).^{14,17-19,21-23,27,28,32,35,36,40,56,67,68,73,77,79,92,99,100}

Acid Phosphatase

Acid phosphatase is a group of enzymes that hydrolyze phosphate esters in an acidic environment. There are erythrocyte and leukocyte isoenzymes that are specific for different cells. Many cell types stain in the ACP cytochemical reaction, including lymphocytes, monocytes, histiocytes, granulocytes (particularly eosinophils), platelets, megakaryocytes, plasma cells, and erythroid precursors.^{8,27} The reaction product gives a diffuse pattern in monocytes, with histiocytes staining more intensely. A single focal or punctate reaction product in the Golgi area occurs in T-lymphocytes.¹⁰⁴ Canine lymphocytes with NK-cell activity also react with the ACP technique as focal-staining (Fig. 144.10).⁷⁶ Approximately one-third of canine NK cells stained positively, and a small percentage remained positive with tartrate incubation.⁵⁴ Tartrate resistance was found in eosinophils and basophils from normal dogs and cats.^{27,28} Tartrate resistance is most helpful in human medicine for recognition of hairy-cell leukemia, a condition not recognized in veterinary medicine.⁵⁷ All ACP isoenzymes are sensitive to tissue processing and heat. Therefore, the ACP technique is recommended only for fresh cytologic or plastic-embedded materials (Fig. 144.13) and not for paraffin-embedded tissues.

Acid phosphatase may be used to detect many different types of blast cells. Specific subtypes of acute myeloid leukemia that display positive activity include myeloblastic leukemia, myelomonocytic leukemia, erythroleukemia, and megakaryoblastic leukemia.^{27,28,67,81} Positive staining has been documented in cases of canine and feline histiocytic sarcoma (malignant histiocytosis).^{22,27,98} Eosinophilic and basophilic differentiation of acute and chronic myeloid leukemia in the cat and dog display positive staining.^{12,28,66} In addition, occasional positive ACP reactions have been docu-

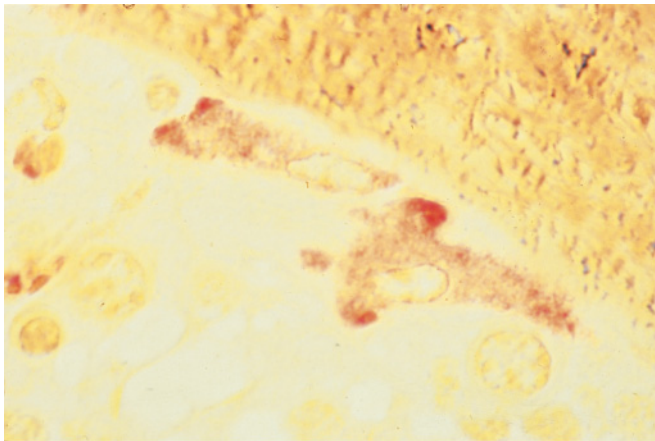


FIGURE 144.13 Bovine bone marrow with diffuse positive ACP reaction in fibrohistiocytic (reticuloendothelial) cells. Plastic-embedded section. $\times 100$ objective.

mented in canine, feline, and porcine mast cell tumors.^{7,28,78} The amoeboid blast cells in a cat that had an acute undifferentiated leukemia had occasional ACP staining.³⁰ There are no known reports of ACP staining in feline cases of monocytic leukemia. Acid phosphatase is used in veterinary medicine primarily to detect focal staining in T-lymphocytes. Some examples of positive ACP staining in lymphoid malignancies may be found in dogs, cats, cattle, sheep, and pigs.^{16,27,28,75,79,82,90,98,105}

Beta Glucuronidase

A hydrolytic enzyme with a mechanism and pattern of reactivity similar to that of the esterases is β G. β -Glucuronidase hydrolyzes the substrate to produce liberated naphthol compounds which subsequently bind to a colored dye. Enzymatic activity is demonstrable in blood lymphocytes, monocytes, and granulocytes (Fig. 144.14). Both monocytes and neutrophils display weak, diffuse, cytoplasmic reactivity, whereas T-lymphocytes produce a focal or distinct granular reaction.⁶⁰ In feline specimens, β G activity appears in monocytes, granulocytes, lymphocytes, and platelets; the strongest reactivity is observed in monocytes.⁹⁶ Weakly positive reactivity has been observed in neutrophils of channel catfish.¹⁰⁷ For human blood, the β G staining procedure appears to have little advantage over NSE in detecting T-lymphocytes.⁶⁰

Diffuse positive staining has been documented in a case of feline acute undifferentiated leukemia.³⁰ β -Glucuronidase is used in veterinary medicine primarily to detect focal staining in T-lymphocytes. Three cats that had large granular lymphocyte lymphoma were noted to have positive staining with β G.^{16,98,99}

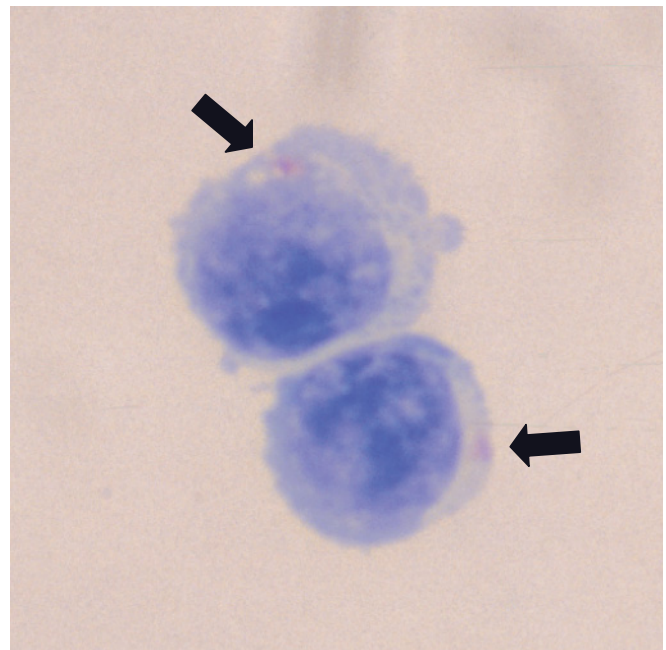


FIGURE 144.14 Focal stain reaction (arrows) with beta-glucuronidase in two canine lymphocytes. $\times 100$ objective.

Miscellaneous Stains

Toluidine Blue

This common histologic stain is helpful for the evaluation of basophil and mast cell populations from many species.^{47,103} Toluidine blue (TB) is a basic dye that reacts with the acid mucopolysaccharides to form metachromatic complexes that appear red-purple. Basophils stain readily in acidic TB but not in neutral TB.⁴⁴

Toluidine blue staining was helpful in the diagnosis of two cases of basophilic leukemia in the dog, and for mast cell identification in tumors from the dog, cat, goat, cow, pig, and horse.^{7,15,52,63,65,66,78,84,86} Several cases of large granular lymphoma or large granular lymphocyte leukemia in the dog, cat, horse, and ferret were evaluated with TB.^{10,16,33,97,98} Of the 13 cases stained in the cat, only one demonstrated a positive reaction.¹⁶

Omega-Exonuclease

Omega exonuclease is an enzymatic staining technique for basophils or mast cells and their precursors in several species, particularly humans, dogs, cats (Fig. 144.7A), and primates such as the Rhesus monkey and the gibbon. Enzymatic activity is not found in normal basophils of the horse, cow, goat, pig, or alpaca.⁴⁷ Positive OEN reactivity produces diffuse, red-brown, granular cytoplasmic deposits. Omega exonuclease is an alkaline phosphodiesterase found within the cytoplasm and not in the specific granules; therefore it is said that OEN activity is more helpful than TB in poorly differentiated or degranulated cells. In addition, it can be helpful to determine the extent of involvement with basophil precursors in cases of acute myeloid leukemia with peripheral basophilia. Omega exonuclease was used for this purpose in one case of basophilia leukemia in the dog.⁶⁶ Also, OEN was used to identify mast cells in tumors (Fig. 144.7B) from a dog and cat.⁷⁸

Synacril Black AN

Synacril black AN application results in selective metachromatic staining of acid mucopolysaccharides found in the megakaryocytic cytoplasm.⁵¹ This substance is a textile dye available commercially as Megacolor from Cytocolor, Inc. After fixation with formaldehyde-acetic acid-ethanol solution, specimens are pretreated with ribonuclease to remove ribonucleic acid. The dye stains the cytoplasm of mature and immature megakaryocytes with a red-violet to violet reaction product (Fig. 144.12A). All other cells are pale yellow to yellow-brown except eosinophils whose granules appear pink. In one report, normal equine megakaryocytes along with normal and neoplastic canine megakaryocytes (Fig. 144.12B) from paraffin-embedded samples stained with a magenta reaction with this dye in an attempt to distinguish megakaryocytic precursors from neoplastic histiocytes.⁶⁹ Immunohistochemical staining using antibodies against human von Willebrand factor (factor VIII-related antigen) and human platelet glycoprotein

(GP IIIa) helped support the characterization of the neoplastic cells in several cases of canine and feline megakaryoblastic leukemia.^{21,67,74}

Acetylcholinesterase

Acetylcholinesterase (ACH) is another enzymatic marker that specifically identifies mature megakaryocytes and platelets from dogs, cats, and rats.^{43,48} In addition, ACH is found also within human and murine erythroid precursors.⁵⁵ Specimens for cytochemical staining are incubated in a medium containing acetylthiocholine iodide as substrate. The subsequent reaction product is brown.

Acetylcholinesterase staining was helpful in the identification of megakaryocytic precursors in canine cases of megakaryoblastic leukemia and essential or primary thrombocythemia.^{18,40}

Luna

Luna is a specific stain for eosinophil granules that can be used to identify atypical granulocytes or blast cells with eosinophilic differentiation. It was helpful in confirming the presence of eosinophilic precursor cells in a case of leukemia in a horse in which there was a predominance of positively stained cells.⁷⁰

SUMMARY

Cytochemical staining is relatively inexpensive and provides a convenient manner to identify the chemical constituents of blood cells and characterize poorly differentiated cells in cases of acute nonlymphoid neoplasia. This procedure is particularly useful in a wide variety of animal species including birds, reptiles, amphibians, fish, and invertebrates.

Mammalian granulocytic precursors may be identified by positive staining with peroxidase, Sudan black B, chloroacetate esterase, leukocyte alkaline phosphatase, acid phosphatase, and periodic acid-Schiff. Mammalian monocytic precursors are detected by a positive reaction with nonspecific esterases that are sensitive to fluoride inhibition. Megakaryoblast cells are recognized by positive staining with nonspecific esterases partially sensitive to fluoride inhibition, periodic acid-Schiff, Synacril black AN, and acetylcholinesterase.

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Electrophoresis and Acute Phase Protein Measurement

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Basis of Electrophoresis	Detecting Acute Phase Proteins
Types of Electrophoresis	Technical Aspects
Zone Electrophoresis	Acute Phase Protein Measurements
Cellulose acetate electrophoresis	C-Reactive Protein
Agarose gel electrophoresis	Serum Amyloid A
Polyacrylamide gel electrophoresis	Haptoglobin
Capillary Electrophoresis	Alpha-1-Acid Glycoprotein
Immunofixation Electrophoresis	Ceruloplasmin
Electrophoretic Patterns in Different Species	APP Measurements in Fluids or Tissues Other than Serum
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Acronyms and Abbreviations

AGE, agarose gel electrophoresis; AGP, alpha-1-acid glycoprotein; APP, acute phase proteins; ASG, acid soluble glycoprotein; CE, capillary electrophoresis; Cp, ceruloplasmin; CRP, C-reactive protein; CZE, capillary zone electrophoresis; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme linked immunosorbent assay; Hp, haptoglobin; HRE, high resolution electrophoresis; SAA, serum amyloid A; SPE, serum protein electrophoresis.

BASIS OF ELECTROPHORESIS

During the past two decades there has been an increase in the application of serum protein separation as a laboratory test for diagnostic purposes in veterinary patients. The serum protein profiles are mostly defined using routine serum protein electrophoresis (SPE), which allows identification of different fractions or regions, each being composed of one or several proteins, with similar electrophoretic mobility.¹ From a general point of view, electrophoresis is used to separate macromolecules from complex mixtures by application of an electric field. Different macromolecules in the mixture will migrate at different speeds, depending on the nature of the gel and the physical-chemical characteristics of the macromolecules. Electrophoretic techniques can be applied to the separation of any macromolecule, including nucleic acids (DNA and RNA), proteins, lipids, and carbohydrates. However, the principles of separation

and matrices used may differ depending on the molecules that need to be separated.⁶

A typical electrophoresis system includes five components:^{10,22} (1) buffer solution (with protein electrophoresis barbital buffer having a pH near 8.6 is the most commonly used); (2) two electrodes of either platinum or carbon, the polarity of which is fixed by the mode of connection to the power supply; (3) the electrophoresis support on which separation takes place; (4) a cover to minimize evaporation and protect the system; and (5) a power supply.

Basically, the electrophoresis procedure comprises two steps: separation and detection. To perform an electrophoretic separation, the electrophoretic cell, support media, and voltage meter are prepared following technique specifications. Sample is then added to the media in a narrow band or spot using the method appropriate to the type of media used. Electrophoresis is conducted for a determined length of time under conditions of

either constant voltage or constant current. After completion of electrophoresis the media is treated to allow visualization of the protein. This procedure involves protein fixation and staining. An exception is capillary electrophoresis (CE) in which proteins are eluted and pass through a detector that directly measures the UV absorbance of the peptide bonds. Once electrophoretic separation and staining are complete, direct densitometry is used to quantify the individual zones, either as a percentage of the total or as absolute concentration if the total quantity of protein is known.

In routine clinical chemistry, the main purpose of performing electrophoresis on body fluids such as serum, urine, and cerebrospinal fluid (CSF) is to separate the constituent proteins into major fractions. Electrophoresis generates an electrophoretogram, a display of protein zones, each separated from neighboring zones on the support material. The different electrophoretic patterns could be then interpreted and abnormal findings correlated with disease states.

TYPES OF ELECTROPHORESIS

Zone Electrophoresis

Zone electrophoresis uses a porous medium to support the buffer and allows migration of charged protein molecules on it.

Cellulose Acetate Electrophoresis

Cellulose acetate has the advantage of being inexpensive, and allows fast electrophoretic separation (20 minutes to 1 hour) and long storage time of the transparent membranes. However, several time-consuming procedures are required, including presoaking before use and clearing of membranes before densitometry. Additionally, high-resolution separation is impossible with cellulose acetate electrophoresis. As a result of these limitations, cellulose acetate has been replaced by other types of electrophoresis in most clinical applications.

Agarose Gel Electrophoresis

Agarose gel electrophoresis (AGE) has been one of the most common procedures in veterinary medicine for analysis of serum proteins in clinical situations. It has several advantages over cellulose acetate:^{10,22} (1) AGE can be automated. Although standard AGE is a labor-intensive procedure, the introduction of prepackaged gels and integration of computers with electronic instrumentation has resulted in the availability of a number of AGE instruments with semi-automated capabilities. Although this has made the technique less labor-intensive, AGE is not amenable to full automation. (2) Variable pore size makes it possible to use AGE for separation of other molecules such as lipoproteins and nucleic acids. (3) AGE has much better resolution than cellulose acetate. In addition, some laboratories are

using an improved method known as high resolution electrophoresis (HRE) that allows the detection of several protein bands that are not well resolved by conventional SPE. High resolution electrophoresis may be particularly useful to detect monoclonal paraproteins but is of limited benefit for analyzing other serum protein patterns, because clinical applications of this information in veterinary medicine are not yet widely understood.¹³ (4) The medium is clear after drying and therefore leads to very sensitive densitometry measurements.

There are disadvantages of AGE, including fragility of the media so that the gel must be handled carefully, and the need for expensive equipment.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel separates more serum protein fractions than the other supporting substances, is less fragile than agarose gel, and is transparent allowing analysis by densitometry. However, the technique is relatively labor-intensive for routine applications and is more used in specialized situations or in research studies where high resolution is desirable.^{5,22}

Capillary Electrophoresis

Capillary electrophoresis (CE) allows high-resolution separation of inorganic and organic ions, including nucleic acids, proteins and peptides. In this system, the classic technique of electrophoresis is carried out in a small capillary tube that serves as the electrophoresis chamber that is connected to a detector and, via buffer reservoirs, to a high voltage power supply. There are several CE modes with different separation methods but capillary zone electrophoresis (CZE) is the most frequently used mode for serum protein electrophoresis.^{11,21} With CZE, separation of the protein fractions occur in free solution in a narrow-bore fused silica capillary that is exposed to a high voltage. When voltage is applied two forces (i.e. electrical field and electro-osmotic force) act in opposite directions on the proteins. First, proteins are attracted toward the anode because a borate buffer pH 10 is used and at this pH proteins are negatively charged. Second, an electro-osmotic force is involved because the empty capillaries have a negatively-charged surface making the buffer flow in the direction of the cathode.

In CZE the force of the electro-osmotic flow surpasses the force of the electrical field; as a result all proteins are carried toward the cathode.² Capillary zone electrophoresis has been proposed as an alternative to conventional AGE in separating human and animal serum proteins^{7,19} because it allows fast protein separation (4 minutes compared to 25 minutes) with good efficiency and resolution, and uses only small amounts of sample. Additionally, the method can be fully automated. Commercial systems equipped with automated sample loading and data analysis are available, giving a significant higher sample throughput.

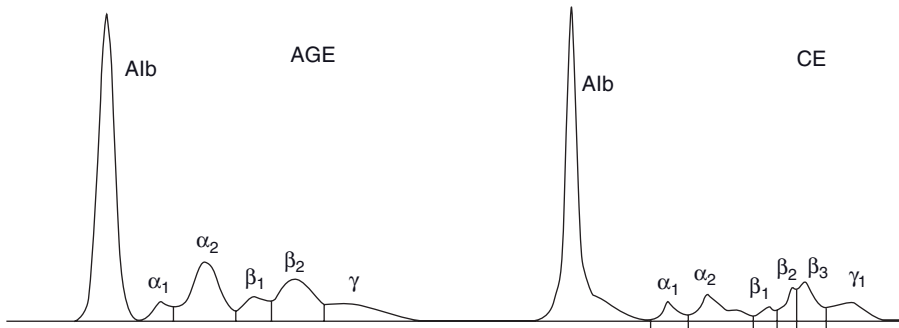


FIGURE 145.1 Agarose gel (AGE) and capillary (CE) electrophoretic patterns of a serum sample from a healthy dog. Notice the larger number of peaks obtained with CE and the better definition of peaks.

Although the initial expense of equipment may be higher than for AGE, the ongoing cost of reagents is relatively small, especially when dealing with a high number of samples.

Immunofixation Electrophoresis

Immunofixation electrophoresis is a two-step process involving AGE followed by specific antibody that is layered over the separated proteins. It is considered the gold standard to determine the presence of monoclonal proteins in cases of increases in gamma fractions found with routine electrophoresis systems.¹⁵

ELECTROPHORETIC PATTERNS IN DIFFERENT SPECIES

Typically proteins are separated into five fractions in humans: albumin, α_1 -, α_2 -, β -, and γ -globulins.³ However, in veterinary medicine more fractions inside β -globulins and even γ -globulins have been described. To identify and separate these fractions in the electrophoretic pattern some rules can be followed: (1) the fractions will appear in the following order: albumin, α -globulins, β -globulins and γ -globulins; (2) albumin is identified as the peak nearest the anode, and usually appears as a high and narrow peak; (3) the midpoint on the horizontal axis of the electrophoretogram is the approximate separation between α -globulins and β -globulins; (4) the separation between α_1 - and α_2 -globulins coincides with the more major valley in the α -globulins fractions; (5) the separation between β - and γ -globulins is established as the most marked valley that appears between the β -globulins and γ -globulins (to determine the end of the β -globulin area it is useful to know that the β -globulin area is of similar size to the α -globulin area); and (6) subfractions in β - and γ -globulins can be established if valleys appear inside these fractions.

Once this initial broad separation into five fractions has been completed, the use of highly sensitive methods such as capillary electrophoresis has allowed identification of selected acute phase proteins (APPs) in humans. For example: α_1 antitrypsin and α_1 acid glycoprotein have been identified in the α_1 globulin fractions, haptoglobin and α_2 -macroglobulin in the α_2 -globulin fraction and transferrin and complement in the β -globulin

fraction.² These APPs could correspond to the subfractions (such as β_1 - or β_2 -globulins) identified in veterinary species. Present automated CE equipment is designed and validated for humans and it does not properly identify fractions and peaks in the animal species. Therefore, manual techniques should be used for animals.

Two main sources of variation in the electrophoretic fraction separation have been identified in veterinary medicine. The protein patterns and numbers of peaks obtained are dependent on the sensitivity of the electrophoresis used. For example, more peaks can be obtained with CE than with AGE (Fig. 145.1). In this sense, it is important to define the electrophoretic fractions and subfractions and establish reference intervals for the equipment and assay conditions used in each laboratory. Even changes in the analytical conditions with the same equipment can influence results.¹⁷ There is also considerable species and breed variation in the number, shape, and size of fractions and subfractions. For example, ruminants have undetectable concentrations of haptoglobin, giving lower values of α_2 -globulin. Variations in serum protein electrophoretic patterns within breeds have been described. For example, Greyhounds have lower serum protein and lower α - and β -globulin concentrations compared with non-Greyhound dogs.⁸ These findings could be influenced by low concentrations of haptoglobin in this breed.

COMPARISON BETWEEN ELECTROPHORETIC METHODS

Correlation of Protein Fractions between Methods

When canine protein electrophoresis was performed by CE and AGE, only albumin and α_2 -globulins had a high degree of correlation.¹⁹ Several reasons could explain the poor correlation of other fractions. In humans, CE gave 2.2 fold higher values for α_1 -globulin compared with agarose electrophoresis.¹⁶ This could be due to the high concentration in sialic acid in α_1 -acid glycoprotein that interferes with the stains used in agarose electrophoresis. The poor correlation found for β -globulin could be due to the different migration of β -lipoproteins. It has been reported to be in the β -fraction in agarose and between α_2 and β -fraction in CE.² In addition, the limits of the fractions were manually defined in both

techniques, producing inaccuracies that could contribute to the poor correlations.

Detecting Monoclonal Peaks

In studies performed in humans, CE seems to have a higher sensitivity for detection of monoclonal peaks than AGE; however, especially at a maximum resolution, the number of false positives is higher in the capillary technique.^{15,16}

Detecting Acute Phase Proteins

A high-resolution CE technique has been described in human to allow separation of α 1-antitrypsin, α 1-acid glycoprotein, and haptoglobin in serum samples. This technique had a strong linear correlation with nephelometric determinations for these proteins.¹⁴

Technical Aspects

Some data are available regarding the influence of several technical aspects in the final electrophoretic results. These data should be viewed with caution, as results may also be dependent on the equipment used and the analytical conditions.

In human studies, inter- and intra-assay precision of less than 5% has been achieved for AGE and less than 4% for CE for all fractions.¹⁶ The manual establishment of the fractions could explain the higher imprecision values in some fractions such as β -globulins when dog samples were analyzed with CZE, although both inter- and intra-assay precisions were less than 14%. In that canine study, the method showed good accuracy when linearity was evaluated by serial dilution of serum samples.¹⁹

Haemolysis and lipemia produced a change in electrophoretogram morphology in canine samples analyzed by CZE, giving an interference peak located in the β region when hemoglobin was increased and in the α 1 region when lipids were increased.¹⁹ Bilirubin produced an increase in albumin and α 1 and a decrease in the α 2 and β 2 fractions. Fibrinogen did not produce any additional peak in CZE, so no differences were found between serum and plasma samples.¹⁹ However, in a recently developed system of CE, fibrinogen was detected in humans.¹⁴

Regarding anticoagulants, plasma samples obtained with heparin, EDTA, citrate, and fluoride can be used for CE assays. In addition, samples can be stored frozen for long periods of time; however, special care should be taken to avoid sample clotting that could occlude the capillary.

ACUTE PHASE PROTEIN MEASUREMENTS

C-Reactive Protein

Serum C-reactive protein (CRP) is generally measured by immunoassays using species-specific CRP antibodies

in several formats such as ELISA, immunoturbidimetric assays adapted for automated biochemical analyzers, slide/capillary latex agglutination tests, or time-resolved fluorometry. Technical improvements are needed to decrease between-run imprecision in some commercial ELISA kits for dogs and pigs.⁴ Commercially available automated turbidimetric immunoassays, designed for human CRP, have been found to be valid for measuring canine and porcine serum CRP concentration.¹² However, in other investigations very weak or negligible cross-reactivity of canine CRP with different anti-human CRP antibodies was found.²⁰ Special care should be taken when using non-species-specific assays for CRP measurements and a complete validation of each batch of antibody is recommended before use in these cases.

Serum Amyloid A

A commercially available ELISA for serum amyloid A (SAA) determination in veterinary species using monoclonal antiserum against human SAA has proven to be useful for canine, feline, and porcine SAA quantification,^{4,18} however, ideally interassay imprecision should be reduced. An automated commercially available human SAA turbidimetric immunoassay has been validated for feline and equine SAA determination,⁹ but does not seem to work in dogs.

Species-specific standards and serum with a high concentration of SAA instead of the purified protein are recommended when the human test is used in animals.

Haptoglobin

Assays for serum haptoglobin (Hp) concentration can be divided into two groups: spectrophotometric and immunoassays. An automated spectrophotometric multispecies assay based on the peroxidase activity of Hp-Hgb complexes, in which interference by serum albumin is eliminated, has been developed into a commercially available kit. This assay was initially developed for Hp measurements in ruminants, so in other species with higher concentrations of Hp, samples should be diluted.⁴ Human automated immunoassays have been validated for Hp measurement in animal species. As indicated previously, these assays will depend on the cross-reactivity of the antiserum with the Hp of the species tested and must be properly validated before use.²⁴

Alpha-1-Acid Glycoprotein (AGP)

Alpha-1-acid glycoprotein (AGP) can be measured by single radial immunodiffusion on agarose gel or by immunoturbidimetric assays. Both methods use species-specific antibody. Alternatively, AGP can be indirectly estimated as seromuconoid or acid soluble glycoproteins (ASGs) by precipitation of the majority of serum proteins by perchloric acid and quantification of the remaining soluble proteins, although in some

species such as pigs it seems that AGP is not a major component of the ASG.²³

Ceruloplasmin

Automated spectrophotometric assays have been developed for ceruloplasmin (Cp) measurements in pigs and dogs based on oxidation of different compounds such as *p*-phenylenediamine and *o*-dianisidine dihydrochloride. The main problem with Cp assays is the lack of available reference materials to standardize Cp concentration (different arbitrary units have been used to report assay results).⁴

APP Measurements in Fluids or Tissues Other than Serum

The use of time-resolved fluorometry has allowed accurate detection of very low concentrations of APP in specimens such as effusions, saliva, or meat juice. Saliva is easy to obtain and sample collection is noninvasive. On the other hand, the possibility of APP measurements in meat juice could be applied to meat inspections.

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Measurement of Serum Iron Concentration, TIBC, and Serum Ferritin Concentration

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Measurement of Serum Iron Concentration
Measurement of TIBC
Measurement of Serum Ferritin Concentration

Acronyms and Abbreviations

ELISA, enzyme-linked immunosorbent assay; TIBC, total iron binding capacity; UIBC, unbound iron binding capacity.

MEASUREMENT OF SERUM IRON CONCENTRATION

Iron is transported in plasma by the iron transport protein apotransferrin. Each apotransferrin molecule has two iron binding sites that bind one Fe^{3+} ion. The apotransferrin- Fe^{3+} complex is called transferrin. Under normal conditions approximately one-third of the iron-binding sites of transferrin are occupied by Fe^{3+} . Serum iron concentration refers to the Fe^{3+} bound to transferrin and does not include iron contained in serum as free hemoglobin.

The most common methods employed to measure serum iron are colorimetric procedures based on the formation and quantitation of a colored iron-chromogen complex. Iron is released from transferrin by strong acidification of the serum by addition of hydrochloric, sulfuric, or trichloroacetic acid (that simultaneously precipitates serum proteins). Fe^{3+} is reduced to Fe^{2+} by addition of ascorbic acid, thioglycolic acid, hydrazine, or hydroxylamine,²¹ and is then complexed with a chromogen that contains the reactive group $-\text{N}=\text{C}-\text{C}=\text{N}-$.⁶ Metal cations are chelated between the two nitrogens forming a colored reaction product. The absorbance of the chromogen at its characteristic wavelength is proportional to the iron concentration. Chromogens commonly used include 4,7-bis(4-phenyl sulfonic acid)-1,10-phenanthroline, sodium salt (bathophenanthroline disulfonate, sodium); 2,4,6-tripyridyl-*s*-triazine (tripyridyl triazine); 3-(2-pyridyl)-5,6-bis(4-phenyl sulfonic acid) 1,2,4 triazine, sodium salt (ferrozine); and 2,6-bis(4-phenyl-2,2-pyridyl)-4-phenyl pyridine (terosite).⁶ The

assay can be performed manually,⁵ by commercial kits, or automated analyzers.

Thiourea and thioglycolic acid are chelating agents used in some assays to prevent copper from complexing with the chromogen and interfering with iron measurement.¹⁴ Minute amounts of iron can be complexed with other serum proteins. Under normal circumstances this amount is clinically insignificant. Some assays use trichloroacetic acid to precipitate serum proteins or dialysis to remove them. Iron released from serum ferritin in cases of hyperferritinemia can elevate the serum iron concentration when measured by some iron assays.²⁰

Serum iron has also been measured by coulometry.¹⁸ The principle of the test is based on development of an electrochemical potential at the interface of a salt solution and an electrode. The amount of current applied to maintain the electrical potential of the electrode at a constant value depends on the electropositivity of the metal being measured and the concentration of the metal. The electropositivity of a metal is a measure of the tendency of a metal to lose one or more electrons. The electropositivity of iron is a known constant value, so the amount of current required is proportional to the iron concentration. The method correlates well with chromogenic methods, has very good accuracy and precision, can be used with micro-samples, and can be automated.¹⁴ Unfortunately, the instrument is no longer being manufactured. Other methods are radiometry and atomic absorption spectrophotometry, but these are rarely used in clinical practice.

Iron is ubiquitous in the environment, so care should be taken to ensure blood collection tubes, glassware, water, and reagents are free of environmental contamination with iron. Hemolysis has little effect on serum iron assays because the iron in hemoglobin is not released by acidification of the serum. However, hemoglobin can cause spectral interference in colorimetric assays, so grossly hemolyzed samples should not be analyzed.⁸ In human medicine, reference intervals can vary substantially between commercial laboratories so it is recommended that laboratories establish their own reference intervals.¹⁰ The same recommendation applies to veterinary samples.

MEASUREMENT OF TIBC

Total iron binding capacity (TIBC) is the amount of iron that serum transferrin can bind when all iron-binding sites are saturated and is, therefore, an indirect measure of serum transferrin concentration. Two methods can be used to measure TIBC.¹⁴ In one method, excess Fe³⁺ in the form of ferric ammonium citrate is added to the serum adjusted to a pH of 8.0 or above with a buffer solution. At this pH, the iron-transferrin complex is stable and transferrin will bind iron until all binding sites are saturated. The excess unbound Fe³⁺ is removed by an ion exchange resin or by addition of light magnesium carbonate (MgCO₃) powder and the iron assay is repeated. The value obtained is the TIBC.

In the second method a known amount of excess iron is added and the pH adjusted to 7.5 or above. At this pH, only the excess free iron can be reduced to Fe²⁺ and react with the chromogen. The iron bound by transferrin remains as Fe³⁺ and gives no color reaction. The unbound iron in the supernatant is measured by the iron method. The concentration of the iron standard minus the unbound iron is called the unbound iron-binding capacity (UIBC), which is used to calculate the TIBC as follows:

$$\text{TIBC} = \text{UIBC} + \text{total serum iron concentration.}$$

Transferrin saturation is the percentage of transferrin iron-binding sites that are occupied. It is calculated as follows:

$$\text{Transferrin saturation (\%)} = \frac{100 \times \text{serum iron concentration}}{\text{TIBC}}.$$

MEASUREMENT OF SERUM FERRITIN CONCENTRATION

Serum ferritin is measured by immunologic methods. In human medicine, immunoradiometric assay, enzyme-linked immunosorbent assay (ELISA), immunochromoluminescent, and immunofluorometric methods are used.¹⁰ Assays to quantitate human serum ferritin are available in kit form and automated immunoassay instruments.

Immunoassays to quantitate human serum ferritin cannot be used to quantify serum ferritin in other species. Quantitative immunoassays must be calibrated with a standard curve of known concentrations of the purified analyte being measured from the species of interest in order to determine the concentration of the analyte in the unknown sample. Although there is serologic cross-reactivity between serum ferritins of some species,¹⁹ in general, serum ferritins tend to be immunologically species-specific.¹³ Therefore, individual assays still must be developed and calibrated for each species.

ELISA tests have been developed for serum ferritin in the dog,⁴ cat,³ horse,^{9,12,17} pig,^{7,16} cow,¹¹ northern fur seals (*Callorhinus ursinus*),² black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceroses,¹⁵ and black and white ruffed lemur (*Varecia variegata variegata*).¹ In all cases, serum ferritin was purified from each species and quantitated by chemical methods. Polyclonal antisera or monoclonal antibodies were raised to the ferritin and used to develop tests for the dog, cat, horse, pig and cattle. In some instances there was sufficient species cross reactivity to allow use of antisera or monoclonal antibodies to serum ferritin of one species to develop an ELISA for another species. Anti-equine ferritin polyclonal antibodies were used for the rhinoceros,¹⁵ mouse monoclonal anti-dog ferritin antibody for the northern fur seal,² and anti-human ferritin polyclonal antibodies for the lemur ELISA test.¹ Each ELISA is calibrated with a standard curve of known concentrations of the ferritin purified from the species being measured. Non-human serum ferritin assays are offered by the Comparative Hematology Laboratory at Kansas State University.

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Molecular Techniques and Real-Time PCR

CHRISTIAN M. LEUTENEGGER

Sample Submission and Preanalytical Considerations
Nucleic Acid Isolation
Reverse Transcription and Generation of cDNA
RNA Applications

Amplification of DNA and PCR in General Terms
Real-Time Quantitative PCR
Sequencing
Applications of Molecular Techniques

Acronyms and Abbreviations

DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetic acid; FCoV, feline corona virus; FeLV, feline leukemia virus; FIV, feline immunodeficiency virus; ³²P, radioactive isotope of phosphorus; PCR, polymerase chain reaction; RNA, ribonucleic acid; RT PCR, reverse transcription PCR.

Over the past 20 years, knowledge of infectious diseases including diagnostic approaches and treatment options has advanced significantly. During this period the discoveries of a number of important human and animal agents, antimicrobials, and vaccines as well as a wealth of improved diagnostic tests have been reported. Despite these advances, infectious diseases have remained a leading cause of morbidity and mortality with resurgence of certain infections combined with new resistance patterns.⁷ Molecular tools are now being used in addition to conventional culture techniques and antibody based tools for identification and diagnosis of infectious diseases.^{13,14} This era of molecular diagnostics for infectious diseases began in 1983 when Dr. Kary Mullis of Cetus Corporation first conceptualized the polymerase chain reaction (PCR).^{17,22} He went on to win the Nobel Prize in Chemistry for this technology in 1992. Since the inception of the PCR technology, infectious disease diagnostics have been at the forefront of molecular medicine. Infectious disease testing is expected to increase in value for veterinary molecular diagnostics.

SAMPLE SUBMISSION AND PREANALYTICAL CONSIDERATIONS

In general, molecular diagnostic laboratories provide strict recommendations for sample collection including shipping instructions. These instructions include specimen type, volume, anticoagulant, and specimen trans-

port, storage, and handling. The sample type is largely influenced by the pathogenesis of the disease and plays a key role in performance and interpretation of test results. Veterinarians are advised to adhere to these recommendations, as the quality of the result is directly related to the quality of the sample and its preservation of the nucleic content. Molecular assays often offer the convenience of using a small minimally-invasive specimen. The diagnosis of herpes viruses is a classical example, where culture and neutralization assays have been largely replaced by PCR testing on a small volume of aspirate or swab from mucosal surfaces.²⁸ Molecular tests can detect the presence of small numbers of organisms; however, the probability of detection increases when a larger volume of specimen is added to the amplification reaction. This reflects, in part, the probability of having any organisms in the tested volume of sample. Because molecular assays do not need viable organisms for testing, more flexibility in specimen transport is possible than for culture methods.

Appropriate specimen collection and transport conditions are important to ensure successful extraction of intact nucleic acid and to prevent pre-analytical cross-contamination.²⁵ Detailed storage and shipping instructions are crucial if RNA pathogens are part of the diagnostic workup. Large animal practitioners should be aware of these recommendations and consider using appropriate cooling containers when samples are collected in the field. Such samples maintain stability for days if stored appropriately in a cooled environment. Short-term freezing of fresh specimens frequently

affects the quality adversely and should be avoided if not otherwise instructed by the laboratory. Sampling and labeling errors, and contamination of sample containers on their outside are among the many pre-analytical variables to consider; they are not unique to PCR but are general to any laboratory diagnostic test.

NUCLEIC ACID ISOLATION

The purification of nucleic acids (DNA and RNA in case of RNA viruses or viability assessments) is a fundamental step in molecular diagnostics.^{2,15,29} The quality and fitness of a molecular diagnostic test is in most part directly dependent on the quality and quantity of nucleic acid. Lysates containing crude total cellular material, including proteins, fats, and nucleic acids, are of limited use for PCR; they contain PCR inhibiting components affecting the limit of detection of the assay.⁸ In most cases, the molecular test loses its sensitivity when performed on cell lysates.

In veterinary molecular diagnostics, the variety of sample types make processing a challenge. Automated or semi-automated processing platforms are rapid, accept sufficient specimen sample volume, and provide more consistent and reproducible results than manual methods. In general, these systems are total nucleic acid extraction systems (i.e. deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are extracted) and support the parallel analysis of DNA and RNA pathogens from the same sample. Commercially available automated systems can reduce hands-on labor requirements as well as the risk of cross-contamination during sample preparation.¹⁵ Most systems can accommodate sample volumes to produce an acceptable limit of detection and can process from 12 to 96 samples in parallel. With automated nucleic extraction systems finding use even in low volume research settings, standardization will become an important factor to increase reliability and acceptability and decrease laboratory artifacts.

REVERSE TRANSCRIPTION AND GENERATION OF cDNA

Reverse transcription (RT) is a process describing the conversion of RNA into complementary DNA or cDNA. This process is necessary for applications where the target (i.e. organism) is RNA. The reverse transcription of RNA into cDNA is a necessary step in PCR because DNA polymerase can not amplify RNA. Depending on the application, a one-tube or a two-tube RT protocol is used. One-tube RT-PCR is the norm for stand-alone RNA virus diagnostics. In such a setup, the RNA is reverse transcribed by an RNA-dependant polymerase into cDNA that subsequently is amplified by a DNA-dependant DNA polymerase present in the same tube. However, if multiple targets (viruses or genes) are analyzed from a single sample, a two-tube RT protocol may be more economical and give more reliable results. In this case, the RNA in a given sample is reverse transcribed in a first reaction tube to generate cDNA; then

the cDNA is used in a second tube for the PCR process; hence the acronym one-tube RT-PCR versus two-tube RT-PCR. An additional advantage of two-tube RT-PCR protocols is more uniform generation of cDNA and in many cases increased yield of cDNA compared to one-tube RT-PCR protocols.

RNA APPLICATIONS

RNA-based applications are expanding rapidly in the field of molecular diagnostics. The simplest application is the diagnostic detection of viral genomic RNA. A variation of this application is the detection of splice variants of RNA or DNA viruses, especially if certain splice variants are associated with a particular disease process. For feline corona virus (FCoV) for example, change in the splice variant pattern is indicative of enhanced viral replication in monocytes leading to feline infectious peritonitis (FIP).²⁶ In feline retrovirus infections (feline leukemia virus [FeLV] and feline immunodeficiency virus [FIV]), both DNA and RNA can be detected simultaneously with molecular methods; while DNA detection confirms infection throughout the entire disease course, quantitative analysis of RNA viral load has implications in early detection of infection and as a prognostic marker.¹¹ Furthermore, in the case of herpesvirus infections, the detection of RNA indicates virus replication in host tissues.¹⁹ This application, therefore can differentiate between latent (DNA only) and lytic infections (RNA as well as DNA detected for glycoprotein genes), as exemplified by equine herpes virus-4 (EHV-4).¹¹

Sets of transcribed genes as indicators of a certain disease or disease states are commonly referred to as biomarkers or gene signatures. In this case, the messenger RNA (mRNA) of genes is targeted, which is a marker for gene activity. These gene signatures are becoming increasingly important in noninfectious and certain infectious human diseases. On a research basis, such gene signatures have also been described in various animals.^{3,6,10,16,20,21} Currently, no gene signatures are available in veterinary molecular diagnostic applications but their potential has been recognized.

As a combination of applications described above, the detection of transcribed genes of pathogens at the RNA level has gained importance in recent years for two reasons. First, RNA applications can be of significantly higher sensitivity compared to DNA-based application, especially if multicopy genes such as ribosomal RNA genes are used as targets.^{1,4,27} Second, it detects transcribed genes implying that the organisms are viable, as opposed to their DNA that is present in both dead and live organisms. This is because dead organisms carry detectable DNA for a long period of time; conversely, transcribed mRNA is very unstable and normally disintegrates within minutes after loss of infectivity. PCR tests targeting RNA therefore avoid the frequently mentioned disadvantage of regular DNA-based PCR, namely that of detecting 'only' dead organisms.

AMPLIFICATION OF DNA AND PCR IN GENERAL TERMS

The process of chromosomal duplication during cell division or generation of mRNA from a particular gene both contain the features of the PCR laboratory technique. In essence, PCR was developed using mechanisms used by nature since the dawn of the single cell organism. However, it required the ingenuity of a researcher to add a few variations, namely, the use of primer pairs spanning a relatively short stretch of DNA, repetitive cycles of amplification, and a heat stable DNA polymerase. The bidirectional amplification in a repetitive mode (PCR cycles) allows exponential amplification of DNA: with a single DNA molecule as starting material, an excess of one thousand billion copies are accumulated during PCR with ideal amplification conditions.

After about 40 cycles of amplification, PCR products can be stained with a fluorescent dye and separated by size in a process called gel electrophoresis. Once separated, the gel is illuminated on a UV table to make PCR bands visible. This process of PCR product detection is referred to as conventional or traditional PCR.

REAL-TIME QUANTITATIVE PCR

Traditional PCR has been replaced with second-generation PCR protocols in most human and some veterinary molecular diagnostic laboratories. There are good reasons for this: traditional PCR has low throughput, high risk of PCR product carry-over and lack of standardization. In addition, assembling of PCR reagents is inefficient and frequently leads to unstable reagent mixes, making running controls and standards in parallel with diagnostic samples a necessity.

Real-time PCR was introduced in the mid 1990s (Applied Biosystems, Foster City, CA) and has moved the traditional PCR application from the analog age into the digital real-time age.¹² In this technique, PCR products, accumulated during PCR, are detected in real-time in each and every PCR cycle. This is accomplished by adding an additional oligonucleotide called a probe labeled with two fluorescent dyes. As long as the probe is in solution, the 3' dye (called the quencher) efficiently absorbs the energy of the 5' dye (called the reporter). Subsequent to the probe's binding to the PCR product, the DNA polymerase recognizes the probe as an obstacle sitting on the strand to be copied and digests the probe with its 5' nuclease activity (the process is also called the 5' nuclease assay); this releases the quencher from physical proximity of the reporter whereby the reporter regains its energy.⁹ Once excited with a laser, the emitted light from the reporter dye now unbound in solution is captured on a charge coupled sensor device and the results recorded in a computer in digital format. Real-time PCR is more robust and more reliable than traditional PCR, has better reproducibility, shorter cycling times, improved sensitivity, and increased specificity, and offers larger throughput by running PCR

reactions on standard 96, 384, and soon 1,536-well microtiter plates. In addition, the problem of PCR product carry-over and the risk for false positive PCR results has been significantly reduced.¹⁸ Pre-mixed reagents with long shelf lives (usually 1 year or longer) at 4°C have significantly increased reproducibility. All of these factors together make real-time PCR a virtually ideal diagnostic tool. Since the mid 1990s, a large variety of detection chemistries, probe varieties, and equipment platforms have been introduced into the market all based on the next generation real-time PCR principle.

SEQUENCING

In May 1975, Frederick Sanger from the Medical Research Council's Laboratory of Molecular Biology in Cambridge presented the first partial DNA sequence.²⁴ He went on to deliver the first complete sequence of a viral genome, the 5,375 base pairs in the genetic code of the bacterial phage phi-X174.²³ The Sanger sequencing method used the enzyme DNA polymerase to make numerous copies of the DNA molecules to be sequenced by incorporating a mix of normal and modified (terminator) nucleotides, each radioactively labeled with ³²P. The normal nucleotides were incorporated sequentially, whereas each incorporation of a terminator nucleotide causes the extension of the DNA copy to stop with a radioactively labeled terminator nucleotide. Four reactions, one for each of the four nucleotides A, T, C, and G were run in parallel. These reactions were then electrophoresed through a high percentage polyacrylamide gel to allow the resolution of fragments in single nucleotide increments. The Sanger sequencing method is still in use today; however, technical advancements have brought dramatic changes. While the human genome project was projected to last 10 years with an estimated cost of \$2.7 billion, sequencing methods have advanced to a state where a single human genome is analyzed within a week with costs at approximately \$50,000.

APPLICATIONS OF MOLECULAR TECHNIQUES

A large number of molecular applications are described to determine genetic (see Chapter 148) and infectious diseases known to be involved in veterinary hematology. Infectious pathogens important in veterinary hematology are summarized in Table 147.1. Hemolytic anemia is the primary manifestation of several rickettsial and protozoal diseases in domestic animals. Red blood cells (RBCs) are directly infected by the hemotropic mycoplasmas, eperythrozoonosis, anaplasmosis, babesiosis, theileriosis, and cytauxzoonosis. In trypanosomiasis and sarcocystosis, hemolytic anemia occurs without direct infection of RBCs by any stage of the organisms. However, infection of RBCs is not necessarily a pathogenic event, as illustrated by several proto-

TABLE 147.1 Infectious Pathogens Causing Red Blood Cell Disturbances In Domestic Animals

Cause	Species	Species	Laboratory Abnormalities
Rickettsial	<i>Eperythrozoon</i>	Swine, llama	Hemolytic anemia, icterus
	<i>Anaplasma marginale</i>	Bovine	Hemolytic anemia
	<i>Anaplasma ovis</i>	Goats, sheep	Hemolytic anemia
	<i>Anaplasma platys</i>		Thrombocytopenia
	<i>Anaplasma phagocytophilum</i>	Many	Granulocytic ehrlichiosis, anemia, thrombocytopenia
	<i>Ehrlichia canis</i>	Dog	Monocytic ehrlichiosis; anemia, thrombocytopenia
	<i>Ehrlichia ewingii</i>		Granulocytic ehrlichiosis, anemia, thrombocytopenia
		<i>Ehrlichia chaffeensis</i> <i>Ehrlichia ruminantium</i>	
Neorickettsial	<i>Neorickettsia risticii</i>	Horse, dog	Monocytic ehrlichiosis; anemia, thrombocytopenia
Mycoplasma	<i>Mycoplasma haemofelis</i>	Cat	Hemolytic anemia
	<i>Cand. Mycoplasma haemominutum</i>		
	<i>Cand. Mycoplasma turicensis</i>		
	<i>Mycoplasma haemocanis</i>	Dog	Hemolytic anemia in splenectomized or immunocompromised dogs
	<i>Cand. Mycoplasma haematoparvum</i>		
Protozoal	<i>Babesia canis</i>	Dog	Hemolytic anemia and thrombocytopenia
	<i>Babesia canis canis</i>		
	<i>Babesia canis vogeli</i>		
	<i>Babesia canis rossi</i>		
	<i>Babesia gibsoni</i>		
	<i>Babesia conradae</i>		
	<i>Babesia cati</i>	Cat	Hemolytic anemia
	<i>Babesia felis</i>		
	<i>Babesia bovis</i>	Cattle	Anemia with hemoglobinuria and cerebral babesiosis
	<i>Babesia bigemina</i>		
	<i>Babesia ovis</i>	Sheep	Intravascular hemolysis
	<i>Theileria parva</i>	Dog	Hemolytic anemia, hemoglobinuria, icterus
	<i>Theileria annulata</i>		Hemolytic anemia
	<i>Theileria lestoquardi</i>	Sheep, goat	Anemia, hemoglobinuria
	<i>Theileria mutans</i>	Cattle	Hemolytic anemia
	<i>Theileria cervi</i>	Deer, elk	Hemolytic anemia
	<i>Cytauxzoon felis</i>	Cat	Nonregenerative anemia, thrombocytopenia, neutropenia with degenerative left shift, icterus
	<i>Trypanosoma</i> sp.	Cattle, horse, swine	Hemolytic anemia
	<i>Hepatozoon canis</i>	Dog	Mild anemia, leukocytosis, neutrophilia
<i>Hepatozoon americanum</i>			
Bacterial	<i>Clostridium perfringens</i> type A	Many	Hemolytic anemia
	<i>Clostridium haemolyticum</i>	Cattle	Acute severe hemolysis
	<i>Leptospira</i> sp.	Many	Anemia, hemoglobinuria, icterus, petechial or ecchymotic hemorrhages
Viral	Equine Infectious Anemia Virus (EIAV)	Horse	Anemia, thrombocytopenia
	Feline Leukemia Virus, FeLV-A	Cat	Anemia
	Feline Leukemia Virus, FeLV-C		Pure red cell aplasia
	Feline Immunodeficiency Virus (FIV)		Anemia, neutropenia, lymphopenia
	Feline Panleukopenia Virus		Pancytopenia, mild anemia
	Canine parvovirus 2 (a, b, c)	Dog	Hypercoagulability
	Canine parvovirus 2	Cat	Lymphopenia

zoa, such as *Eperythrozoon*. The diagnostic challenge for hemoparasites occurs because of their variable numbers in circulation and their inconsistent detectability by light microscopy. Parasites exceeding 1,000 parasites/ μL

(corresponding to 0.1% infected RBCs) can be detected by microscopy. PCR, however can detect 1 parasite/ μL .⁵ However, many other infectious agents often go undetected in blood smears because of their size (viruses,

bacteria) or low numbers. Serologic assays are not able to differentiate between past exposure, chronic carrier state and active infection, and are further limited by cross-reactivity between species and false-negative results during early phase of infection. The use of molecular tools, particularly quantitative real-time PCR, has proven helpful in the diagnosis of acute anemias for many applications.

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Genetic Evaluation of Inherited and Acquired Hematologic Diseases

CHRISTIAN M. LEUTENEGGER and URS GIGER

Patterns of Inheritance for Genetic Diseases

- Autosomal Dominant Disorders
- Autosomal Recessive Disorders
- X-Chromosomal Recessive Disorders

Genetic Analysis

- Linkage Studies
- Genome-Wide Association Analysis (GWAA)

Epigenetics

Detecting Mutations in Individual Patients

- Single Nucleotide Polymorphisms (SNPs)
- Deletion/Insertion
- Gene Duplication

Genomics and Proteomics

Expression Profiling

Cytogenetics

Current Genetic Testing

Acronyms and Abbreviations

BAC, bacterial artificial chromosome; CGH, comparative genomic hybridization; CLAD, canine leukocyte adhesion deficiency; DNA, deoxyribonucleic acid; FISH, fluorescent in situ hybridization; FXI, coagulation factor XI; GWAA, genome-wide association analysis; HLA, human leukocyte antigen; ITGB2, integrin B2; LD, linkage disequilibrium; MHC, major histocompatibility complex; miRNA, micro RNA; monogenic, pertaining to or influenced by a single gene; mRNA, messenger RNA; MTLs, monogenic trait loci; PCR, polymerase chain reaction; QTL, quantitative trait loci; RFLP, restriction fragment length polymorphism; SINE, short interspersed nucleotide element; SNP, single nucleotide polymorphism.

Domestic animals provide unique opportunities for exploring genotype-phenotype relationships, due to their history of selective breeding in large population sizes. This enables powerful genetic and statistical analysis. No other model organisms have been genetically modified to the same extent as domestic animals. Animal domestication has a long history (10,000 years) compared with the short time of studying experimental organisms. The selective breeding of certain traits has led to dramatic changes in growth patterns, behavior, fertility, metabolism and resistance to various infectious pathogens. This has been accomplished by altering the frequencies of mutations with resultant phenotypic effects, some of which pre-date domestication. During the past century, animal breeding and selection techniques dramatically changed. Increasingly, phenotypic data have been collected from large numbers of progenies from each potential breeding animal. These data, together with information on

genetic relationships, allow accurate prediction of the ability to transmit favorable allelic variants. Because of the deep screen of these selection criteria, deleterious mutations are rarer than in model organisms such as the mouse. In fact, domestic animals are rather poor models to study deleterious mutations because of the strong selection against them. The selection screen is enriched for mutations with favorable phenotypic effects on the traits under selection (e.g. milk production), but with no or mild deleterious effects on other traits.

The almost bewildering diversity in size, form, color, and behavior of domesticated animals is most pronounced in the dog. An important explanation as to why the dog exhibits more phenotypic diversity is its role as a pet, rather than as a farm animal bred for fitness and high production efficiency. Because of these differences, deleterious mutations have accumulated in certain breeds more rapidly than in others. In particular

in the dog, there is a considerable amount of genetic drift due to founder effects and small effective population sizes. This leads inherently to large haplotype blocks and homozygosity of recessive disorders in certain breeds.²¹ In general, however, it is a common misconception that domesticated animals are highly inbred. Most populations of domestic animals show low levels of inbreeding with the exception of certain breeds of cats and dogs, where strong selection for phenotypic traits (e.g. coat color) in small breeding populations increases homozygosity for certain genetic mutations.

A growing number of monogenic (i.e. pertaining to one gene) disorders in the dog have been characterized at the molecular level (for a comprehensive list, visit the OMIA database at <http://omia.angis.org.au>). An example of a monogenic dog genetic disease that has developed into a useful model for a human disorder is canine leukocyte adhesion deficiency (CLAD). Initially this disease occurred with high frequency in Irish setters (see Chapter 42).^{8,18} The disease, due to defective expression of leukocyte integrins, causes severe recurrent bacterial infections. With a colony of dogs segregated for this mutation, novel hematopoietic therapies for the treatment of this severe immunodeficiency were tested. It was shown that CLAD is treatable by either nonmyeloablative hematopoietic stem cell transplantation from healthy MHC-matched dogs or by ex vivo retroviral-mediated hematopoietic stem cell gene therapy.^{1,2}

The progress of genomics in domestic animals has been mainly hampered by lack of financial resources. However, with the release of the first human genome and genomes and single nucleotide polymorphism (SNP) maps from animals, combined with significant technological advancement, this situation is now rapidly improving.^{11,20} The first draft genome of the dog was released in 2003 followed by a dense SNP map in 2005.^{9,10} The chicken genome sequence was released in 2004.^{6,23} The cattle physical genome was published in 2007, as well as a draft genome and SNP map for the horse and cat.^{12,13,15} The pig draft genome, initiated at the Wellcome Trust Sanger Institute, aims for a 6× coverage with anticipated completion by the end of 2009 (Table 148.1).¹⁴

PATTERNS OF INHERITANCE FOR GENETIC DISEASES

Knowledge of the definitions for a gene, allele, and haplotype can aid in the understanding of patterns of inheritance and in the understanding of genetic analysis. A gene is defined as follows: a DNA segment that contributes to phenotype/function; a trait, any genetically determined characteristic; also, the condition prevailing in the heterozygous state of a recessive disorder. An allele is defined as one of two or more alternative forms of a gene at corresponding sites (loci) on homologous chromosomes, which determine alternative characters in inheritance. A haplotype is a group of alleles of linked genes (for example, the human leukocyte antigen [HLA] complex) contributed by either parent. There are two rules that are important for understanding the inheritance of genetic disorders: (1) whether the disorder occurs with *one* copy of the mutated gene or *two*, and (2) whether the genetic mutation occurs on one of the *sex* chromosomes (X or Y), or on one of the *autosomes*. These rules combine to make patterns of autosomal dominant, autosomal recessive, and X-chromosomal recessive and rarely dominant inheritance. It is important to recognize that the terms dominant and recessive, according to the Mendelian concept, refer to phenotypic presentations of heterozygous and homozygous animals for a particular trait.

Autosomal Dominant Disorders

In autosomal dominant disorders, the mutated gene occurs on an autosome; one affected copy is deleterious and causes manifestations of the genetic disorder. Males and females are equally likely to inherit the genetic mutation and be affected by it. Offspring of an animal with an autosomal dominant disorder have a 50% chance of inheriting the disorder. Phenotypic expression can be variable and homozygosity may be lethal.

Autosomal Recessive Disorders

In autosomal recessive disorders, the mutant allele also occurs on an autosome; but both alleles of the gene need to be affected for the genetic disorder to become

TABLE 148.1 Genome Resources of Domestic Animals and Human^a

Species	Release Year	Database Resource	Reference
Human	2000	http://www.pubgene.org	11, 20
Dog	2003	http://www.dogmap.ch/	9, 10
Chicken	2004	http://poultry.mph.msu.edu/	23
Cattle	2007	http://www.bovinegenome.org	15
Horse	2007	http://www.broad.mit.edu/mammals/horse	13
Cat	2007	http://home.ncifcrf.gov/ccr/lgd/comparative_genome/catgenome/index_n.asp	12
Pig	In progress	http://www.piggenome.org/	14

^aDraft and full genome sequences for all species including human are also available at <http://www.ncbi.nlm.nih.gov>.

phenotypically manifest. Carriers are healthy animals with one mutant allele and are important because they can pass on the genetic mutation to offspring. Offspring of parent animals each carrying a mutant allele have a 25% chance of inheriting both mutations and having the disorder, a 50% chance of inheriting only one copy of the mutant gene (thus becoming an asymptomatic carrier), and a 25% chance of not inheriting the mutation (clear).

X-Chromosomal Recessive Disorders

In X-chromosomal recessive disorders, the mutant gene occurs on the X chromosome. As males have only one X chromosome, a mutant allele on the X chromosome is enough to cause a disorder. Females have two X chromosomes; therefore a mutant allele on one X chromosome has minimal effects on a female because of the wild-type copy of the same gene on the other X chromosome (i.e. a female with one gene mutation on one X chromosome would be a carrier). On average, 50% of her male offspring will inherit the mutation and develop the disorder, and 50% of her female offspring will inherit the mutation and become carriers.

GENETIC ANALYSIS

Linkage Studies

Classical linkage analysis is used to determine the arrangement of genes on the chromosomes of an organism. By tracing how often different forms of two variable traits or markers are co-inherited, one can infer whether the genes for the traits are on the same chromosome (called linked genes/markers), and if so, one can calculate the genetic distance between loci of the linked genes. The order of and pairwise distances between the loci of three or more linked genes are displayed as a genetic-linkage map.

The genetic signal in a linkage experiment comes from tracing the inheritance of gametes transmitted from heterozygous parents to their progeny. Monogenic traits with a direct relationship between genotype and phenotype are easy to deduce. A panel of a few hundred highly informative markers is sufficient for the genome-wide scan, which then is followed with fine mapping of the target region. The last step involves traditional sequence analysis of the chromosomal region surrounding the marker for potential candidate genes (positional candidate gene approach). The access to draft genome sequences and high-density SNP maps is of significant importance for linkage analysis and paves the way for genome-wide association analysis (GWAA).

Genome-Wide Association Analysis (GWAA)

Linkage analysis of multifactorial traits controlled by quantitative trait loci (QTL) is much more challenging

than linkage mapping of monogenic trait loci (MTLs). This is because the phenotypic effect of each involved locus is small or moderate with no simple one-to-one relationship between genotype and phenotype.

The flow of positional identifications of genes underlying simple and complex genetic traits has been significantly accelerated since the generation of the draft genome sequence and a dense SNP map in the dog in 2005.^{10,22} Because of this, the current trend is to replace the linkage approach by genome-wide association analysis in order to circumvent some of the problems associated with classical linkage analysis. First, there is no need to collect pedigrees because the association analysis is based on case versus control material. Ideally the cases should be as unrelated as possible, while the controls need to be well matched. Second, because of the availability of high resolution SNP maps linked to genome sequences, identification of the causal genes is possible in most cases. Association mapping is based on the presence of linkage disequilibrium (LD) between markers and causal polymorphism. The number of markers required for a GWAA is thus dependent on the length of the haplotype blocks (i.e. region of genome with complete LD information). The length of a haplotype block from the HapMap project is estimated to be about 10kb: as a consequence, genome scans using 100,000 SNPs tested on a few hundred cases and controls are the basis to resolve multifactorial traits.⁹ Because of the existence of high density SNP maps and availability of SNP screening tools (for example SNP arrays), this approach to resolve multifactorial traits has become feasible for domestic animals.

For monogenic traits, GWAA will be an extremely powerful approach for gene mapping: for a simple recessive trait, a sample size of 10 affected animals and 10 controls screened using a sufficiently dense set of SNPs (designed in accordance with the LD pattern) may be sufficient for an initial mapping. This method has been demonstrated for the fibroblast growth factor gene mutations that predispose to the developmental disorder of the dermoid sinus in Rhodesians and Thai Ridgeback dogs.⁴

EPIGENETICS

Epigenetics describes heritable phenotypic changes not explainable solely by genetic influences but also by additional non-genetic influences.¹⁹ Phenotypic changes remain throughout the cell's life and may last for multiple generations without a change in the DNA sequence. The field of epigenetics could explain phenotypic variation not found in pure genetic studies. Genetic statisticians can develop tools for analyzing massive amounts of genotypic data in conjunction with environmental factors, thereby allowing for the identification of risk genotypes as well as gene-gene and gene-environment interactions. Thus risk batteries of genetic variation and environmental factors can be developed

for determination of disease risks associated to genetic diseases.

DETECTING MUTATIONS IN INDIVIDUAL PATIENTS

A number of genetic testing methods are available in reference laboratories. Among the most frequently used tests are polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), and more recently array-based detection systems.

Single Nucleotide Polymorphisms (SNPs)

Point mutations or single nucleotide polymorphisms cause at least one or more of three outcomes: (1) missense mutation, where a point mutation results in the exchange of one amino acid for another in the final protein product; (2) nonsense mutation where a point mutation results in no or a truncated protein product by creating a premature stop codon; and (3) splice mutation where a point mutation alters the protein product by preventing normal post-transcriptional processing of RNA. An example for a missense mutation is the factor VII deficiency in the Beagle, Scottish Deerhound and Alaskan Klee Kai dog, where a G to A missense mutation in exon 5 results in substitution of glycine 96 (GGA) to glutamic acid (GAA) in the second epidermal growth factor-like domain.^{3,7} An example of a nonsense mutation is phosphofructokinase deficiency in English Springer Spaniels. An example of aberrant splicing resulting in a premature termination of transcription is hemophilia A (factor VIII deficiency) in dogs.⁵ Beside disease-causing point mutations there are many other SNPs that do not affect the function of a protein and are not within a gene, but may serve as markers for GWAA.

Deletion/Insertion

A deletion or insertion is a loss or gain of DNA sequence information that can range from a single base pair within a gene to a large part of the chromosome. A deletion or insertion may cause a frameshift mutation: a change in the reading frame of the gene (this occurs if the number of base pairs in the deletion or insertion is not a multiple of three). In most cases, the altered reading frame results in a truncated or elongated protein with affected protein function.

An example for an insertion is the coagulation factor XI (FXI) deficiency in Kerry blue terrier dogs. Exon 7 of the FXI gene shows a short interspersed nucleotide element (SINE) insertion, disrupting the reading frame.¹⁷

Gene Duplication

Gene duplication or gene deletion is the repetition or deletion of an entire gene during meiosis due to unequal

crossing over or mispairing of chromosomes/sister chromatids. An example of gene duplication would be fibroblast growth factor genes in Rhodesian and Thai Ridgeback dogs, that cause hair ridges and a disposition to dermoid sinus.⁴

GENOMICS AND PROTEOMICS

Functional genomics bridges over into proteomics. Proteomics studies protein function in the context of the entire organism. The study of small peptides and the receptors with which they interact is called peptidomics.

EXPRESSION PROFILING

A variation of genetic testing that targets identified genetic mutations is the study of the transcriptome of an organism. Gene expression profiling by microarray technologies has been successfully applied to study the transcriptional changes that occur in diseased versus normal tissues, such as heart, vessels and blood cells in different cardiovascular disorders. The aim of these studies is to unravel the complex molecular picture underlying a particular pathophysiology. Gene expression studies may provide a finer molecular classification of patients with diseases (genetic or not) and indicate new markers useful for prognostic and therapeutic strategies. In addition to the study of expressed genes (messenger RNA [mRNA]) themselves, the study of the post-transcriptional regulation of gene expression by micro RNA (miRNA) adds a layer of complexity and opportunity. Relatively few regulatory miRNA molecules have been identified in humans (678 to date) compared to expressed genes (approximately 30,000).¹⁶ (For further information see miRBase, v12.0 at <http://microrna.sanger.ac.uk/cgi-bin/sequences/browse.pl>.) Many miRNA are highly tissue- and cell-specific and the signal is not confounded by the large pool of “irrelevant” genes.

CYTOGENETICS

Cytogenetics is the study of the structure and function of the cell, especially the chromosomes. It includes routine analysis of G-banded chromosomes, other cytogenetic banding techniques, as well as molecular cytogenetics such as fluorescent in situ hybridization (FISH; Fig. 148.1) and comparative genomic hybridization (CGH). Both techniques are aimed at detection of chromosomal imbalances. FISH can be used to detect and localize the presence or absence of specific DNA sequences on chromosomes. It uses fluorescent probes that bind to only those parts of the chromosome with which they show a high degree of sequence similarity. Fluorescence microscopy is used to find where the fluorescent probe binds to the chromosomes. FISH is often used for finding specific features in DNA for use in

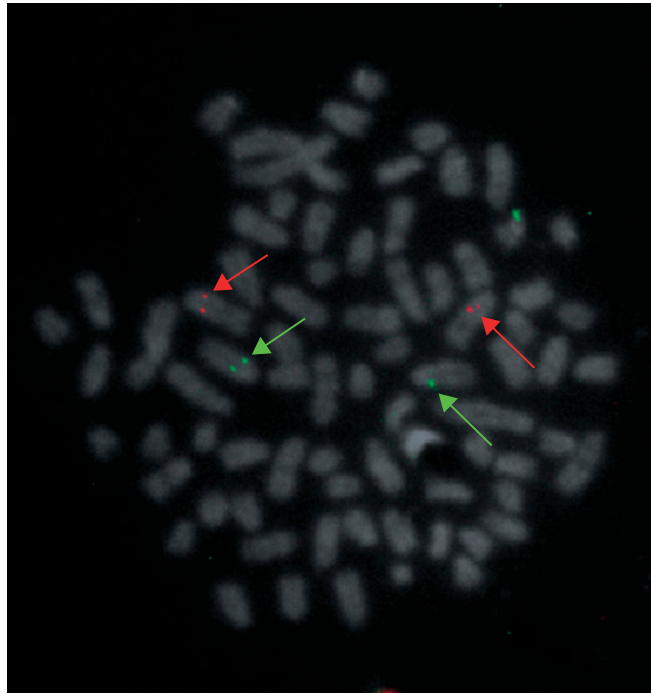


FIGURE 148.1 Results of fluorescence in situ hybridization (FISH) analysis of cultured peripheral-blood lymphocytes using the VIM-biotin (green) and Versican digoxigenin (red) labeling of CFA2 and CFA3 chromosomal pairs, respectively.

TABLE 148.2 Molecular Genetic Tests Available for Hereditary Hematologic Diseases in Dogs and Cats

Genetic Disorders	Affected species and breeds	Laboratory
Erythrocytic disorders		
Pyruvate Kinase (PK) deficiency	Abyssinian, Somali, DSH cats	1,2,11,13
	Basenji	1,4,10,11,13
	Beagle	1
	West Highland white terrier	1,4,5,10,11,13,14
Phosphofructokinase (PFK) deficiency	American Cocker Spaniel	1,3,4,5,10,14,13,14
	English Springer Spaniel	
Feline blood type B	Cats	2,7,9
White blood cell disorders		
Canine leukocyte adhesion deficiency (CLAD)	Irish setter, Red & white setter	3,5,9,11,13
X-linked severe combined immunodeficiency (SCID)	Basset Hound, Welsh Corgi	1,11,13
Mucopolysaccharidosis Type I, II, IIIB, VI, VII	Several canine and feline breeds	1
Cobalamin malabsorption	Australian shepherd, giant schnauzer	1
Cyclic hematopoiesis	Collie	4,10,11
Bleeding disorders (platelets, plasma coagulation proteins)		
Factor VII deficiency	Alaskan Klee Kai, Beagle, Scottish Deerhound	1,4
Factor XI deficiency	Kerry Blue terrier	1
Glanzmann thrombasthenia	Great Pyrenees, Otterhound	6
Hemophilia B	German Wirehaired Pointer	8
Thrombopathia	Basset Hound, Landseer, Spitz	6
Trapped Neutrophil Syndrome	Border Collie	12
Von Willebrand's Disease	Several dog breeds	4,5,11,13

¹PennGen – University of Pennsylvania

²Veterinary Genetics Laboratory – University of California, Davis

³Optigen

⁴VetGen

⁵Animal Health Trust

⁶Boudreaux Lab – Auburn University

⁷CatGenes

⁸Comparative Coagulation Laboratory – Cornell University

⁹Genomia s.r.o.

¹⁰HealthGene

¹¹Laboklin

¹²University of New South Wales

¹³IDEXX Laboratories Inc., VetMed Labor

¹⁴Veterinary Diagnostics Center

genetic counseling, medicine, and species identification. FISH can also be used to detect and localize specific mRNAs within tissue samples. In this context, it can help define the spatial-temporal patterns of gene expression within cells and tissues.

In CGH, two genomic DNA samples are simultaneously hybridized in situ to normal metaphase spreads, and detected with different fluorophores. The intensity ratio of the two fluorescence signals gives a measure for the copy number ratio between the two genomic DNA samples. Comparative genomic hybridization can be either performed on metaphase chromosomes or using large pieces of cloned genomic DNA (BAC) as an array-CGH.

CURRENT GENETIC TESTING

An ever-expanding number of genetic tests are becoming available for screening of genetic diseases. A partial listing of the molecular genetic tests for hematological diseases available for dogs and cats is provided in Table 148.2.

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