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Preface

It all began in a highly intertwined way. The golden age of medical microbiology, which had its height just before the turn of the 19th century not only witnessed the discovery of numerous medically important pathogens, but was also the cradle of immunological research. R. Koch discovered *Bacillus anthracis* and *Mycobacterium tuberculosis*, and was also the first to describe delayed type hypersensitivity reactions to bacterial products. The work of L. Pasteur was both instrumental in establishing germ theory, and also laid the basis for rational vaccine development. E. Behring and S. Kitasato were not satisfied by their success in obtaining pure cultures of *Clostridium tetani*, and went on to develop vaccines against tetanus. Similarly, the seminal discoveries by E. Metchnikoff of phagocytosis and by J. Bordet of complement-mediated lysis, and the development of the side-chain theory by P. Ehrlich, to name but a few, were at the very interface between bacteriology and immunology. Thereafter, however, medical microbiology and immunology went their own ways.

After a period of quiescence, medical microbiology has been revitalized by its amalgamation with molecular genetics and cell biology, to embark towards an understanding of the molecular cross-talk between the host cell and the microbial pathogen. Immunology dramatically broadened its scope from pure antimicrobial defence towards general topics concerned with its role in homeostasis and pathology within the mammalian organism. Today, the majority of immunological subdisciplines have become virtually independent of their roots. Because of its broad scope, immunology has had to develop various methodologies of its own in addition to assimilating strategies from other fields. Immunology has been at the forefront in the development of several techniques of general importance. With the discovery of monoclonal antibody techniques, ELISA and ELISPOT assays for the detection of molecules and fluorescence-activated cell sorting (FACS) systems for the detection and separation of cells have gained general importance. Equally important is the assimilation of cell biology and molecular genetic techniques. Therefore, it is hardly possible for immunologists to master such a broad array of methodologies.

The need for comprehensive manuals on immunological methods has been fulfilled by several large volumes, including the *Immunology Methods Manual* (I. Lefkovits, Academic Press, London, 1997) and *Current Protocols in Immunology* (J. E. Coligan, A. Kruisbeek, D. H. Margulies, E. M. Shevach and W. Strober, Wiley, Chichester, 1995). These are complemented by more specific books for immunologists and for scientists working in related fields, such as *Antibodies – A Laboratory Manual* (E. Harlow and D. Lane, Cold Spring Harbor Laboratory Press, New York, 1988), the *Laboratory Manual on Manipulating the Mouse Embryo* (B. Hogan, R. Beddington, F. Constantini and E. Lacy, Cold Spring Harbor Laboratory Press, New York, 1994) and, last not least, the famous standard work on *Molecular Cloning* (J. Sambrook, E. F. Fritsch and T. Maniatis, Cold Spring

Harbor Laboratory Press, New York, 2nd edition, 1989). However, whilst being invaluable for immunologic laboratories, these manuals are often too broad for the microbiologist interested in using immunological methods to analyse host–pathogen relationships. It is these scientists for whom the present manual has been devised.

Accordingly, in preparing this volume, emphasis was placed on selection with the risk of bias. Sophisticated immunological techniques, as well as those mostly used in unrelated fields, such as tumour immunology or transplantation immunology, were omitted on purpose. Similarly, general techniques stemming from other fields, but now used widely in immunology, were omitted because excellent manuals are already available, and the reader is referred to those. These include general molecular cloning and cell biology techniques, as well as immunological techniques of broader application, such as the generation of monoclonal antibodies.

On which methodologies then did we focus? First, emphasis was placed on immune responses to bacteria, although many of the techniques described are equally applicable to antiviral and antiprotozoal immunity. Secondly, focus has been directed towards T cells, macrophages and cytokines. The T lymphocyte is the central regulator of the anti-infective immune response and the enormous increase in our knowledge about this cell has only been made possible by the recent development of appropriate techniques. T lymphocytes rarely combat infectious agents directly, but rather do so with the help of macrophages and the dialogue between these cells is mediated by cytokines. Equally large space, therefore, has been reserved for the characterization of cytokines and professional phagocytes. Although *in vitro* analyses are indispensible in investigating the immune response against infectious agents, *in vivo* studies remain of critical importance. In particular, evaluation of immunization strategies (such as vaccination) is impossible without appropriate *in vivo* models. With the advent of transgenic and gene deletion mouse mutants, novel strategies for characterizing the role of defined molecules and cells in the *in vivo* setting became feasible. Therefore, we considered it essential to include chapters dealing with various aspects of experimental animal models.

We hope that this manual fills the gap between immunology and microbiology and helps to re-establish a closer relationship between the two disciplines. After all, infection is the outcome of the cross-talk between prokaryotic and eukaryotic cells, and in mammals the immune system has been given the task of being the major player.

We wish to cordially thank the care given by the editorial staff at Academic Press, particularly Tessa Picknett and Duncan Fatz, as well as our secretaries, Rita Mahmoudi and Constanze Taylor, for their great dedication. Last, but not least, we are grateful to all our colleagues who, by contributing to this manual, have generously let us share their extraordinary expertise.

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Introduction: The immune response to infectious agents

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INTRODUCTION

It is the task of the immune system to protect the host against invading infectious agents and thereby to prevent infectious disease. A plethora of microbial pathogens exists (i.e. viruses, bacteria, fungi, parasites and helminths) that have exploited strategies to circumvent an attack by the immune system. Conversely, the immune system has evolved to provide appropriate defence mechanisms at various levels of 'non-specific' (innate) and 'specific' (adaptive) immune responses. In many instances, an appropriate immune response to an infectious agent requires reciprocal interactions between components of the innate and the adaptive immune systems.

The various micro-organisms have developed different strategies to invade their host. Viruses make use of the host cell's machinery for replication and are thus intracellular pathogens. Helminths, the other extreme, are large organisms that cannot live within host cells, but rather behave as extracellular pathogens. In between are bacteria, which, depending on the species, live within or outside host cells, and protozoa where the extracellular or intracellular localization may depend on the stage of their life cycle.

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The successful combat of an invading infectious agent largely depends on the host's capacity to mount an appropriate protective immune response. As a consequence, the analysis of such interactions between host and invading micro-organisms requires a broad spectrum of immunological methods. This book presents a collection of such methods, which are particularly useful for the *ex vivo* and *in vitro* analysis of murine and human immune responses towards infectious agents. In this introductory chapter, a brief overview of the immune defence mechanism against micro-organisms is given, to provide the reader with a guide to the subsequent chapters.

◆◆◆◆◆ THE INNATE IMMUNE SYSTEM

Several defence mechanisms exist that are ready to attack invading micro-organisms without prior activation or induction. They pre-exist in all individuals and do not involve antigen-specific immune responses. Hence they are referred to as components of the innate immune system. Among these components, the granulocytes, macrophages and their relatives play an important role, especially during the early phases of the immune response. Surface epithelia constitute a natural barrier to infectious agents. Apart from the mechanical barrier, surface epithelia are equipped with additional chemical features that help to restrain microbial invasion. Depending on the anatomical localization, such factors include fatty acids (skin), low pH (stomach), antibacterial peptides (defensins, intestine) and enzymes (e.g. lysozyme, saliva). Once the pathogen has crossed the protective epithelial barrier, cellular effector mechanisms are activated. Granulocytes and mononuclear phagocytes represent the most important effector cells of the anti-infective immune response. The group of granulocytes comprises neutrophils, eosinophils and basophils, all of which possess high anti-infective activity. Neutrophils phagocytose microbes and can subsequently kill them. By means of Fc receptors for immunoglobulin G (IgG) and complement receptors, phagocytosis of microbes coated by antibodies or complement breakdown products is improved. Eosinophils and basophils primarily attack extracellular pathogens, in particular helminths, by releasing toxic effector molecules. Growth and differentiation of eosinophils are controlled by interleukin-5 (IL-5) and that of basophils by IL-4. These cell types express Fc receptors for IgE, which provide a bridge between host effector cells and helminths.

The mononuclear phagocytes comprise the tissue macrophages and the blood monocytes. After activation by cytokines, particularly γ-interferon (IFN-γ), mononuclear phagocytes are capable of killing engulfed micro-organisms. However, in their resting stage, macrophages have a low antimicrobial potential, and thus are often misused as a habitat by many bacteria and protozoa. Killing and degradation of these intracellular pathogens by activated macrophages is achieved by a combination of different mechanisms. The most important ones are:

- Activated macrophages produce toxic effector molecules, in particular reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI), which often synergize in killing various intracellular bacteria and protozoa. Although RNI production is the most potent anti-microbial defence mechanism of murine macrophages, its production by human macrophages is still the subject of debate. However, an increasing amount of data supports RNI production by human macrophages during infectious diseases (MacMicking *et al.*, 1997).
- Soon after engulfment of microbes, the phagosome becomes acidic and subsequently fuses with lysosomes. Lysosomal enzymes have an acidic pH optimum, and thus express high activity within the phagolysosome. These lysosomal enzymes are primarily responsible for microbial degradation (Finlay and Cossart, 1997).
- Both the intracellular pathogen and the host cell require iron. Therefore, depletion of intraphagosomal iron reduces the chance of intracellular survival for various pathogens (Payne, 1993).
- Tryptophan is an essential amino acid for certain intracellular pathogens, such as *Toxoplasma gondii*. Accordingly, rapid degradation of this amino acid impairs intracellular replication of susceptible pathogens (Pfefferkorn, 1984).

Intracellular pathogens have developed various evasion mechanisms that prolong their survival inside macrophages. Some even persist within activated macrophages, although at a markedly reduced level. *Listeria monocytogenes* and *Trypanosoma cruzi* egress from the phagosome into the cytosol, thus escaping intraphagosomal attack (Portnoy *et al.*, 1992). Several intracellular pathogens, such as *Mycobacterium tuberculosis*, remain in the phagosome. However, they prevent phagosome acidification and subsequent phagosome–lysosome fusion. To compete for the intracellular iron pool, some pathogens possess potent iron acquisition mechanisms and, to avoid killing by ROI or RNI, several microbes produce detoxifying enzymes. For example, catalase and superoxide dismutase directly inactivate ROI and indirectly impair RNI effects (Kaufmann, 1993).

In summary, living within macrophages provides a niche that protects intracellular pathogens from humoral attack. Yet, once activated, macrophages are capable of eradicating many intracellular pathogens and of restricting growth of more robust ones. Such pathogens may persist for long periods of time, thus causing chronic infection and disease. In addition to their role in the non-specific anti-infective host response, macrophages contribute to the specific immune response against microorganisms. Therefore, methods of evaluating the functional capacities of murine and human macrophages will be described in the chapters by Haworth and Gordon (Section II) and Krause *et al.* (Section III), respectively. Microbial degradation within macrophages delivers pathogen-derived antigenic fragments (peptides), which enter antigen-processing pathways and lead to the cell-surface expression of 'foreign' microbial antigens in the context of appropriate major histocompatibility complex (MHC) molecules (Germain and Margulies, 1993; Cresswell, 1994; York and Rock, 1996). Such antigenic peptides presented by MHC class I or

class II molecules can then activate specific T lymphocytes. As discussed by De Groot and colleagues (Section I), the determination of MHC-binding peptide motifs of immunodominant antigens from infectious micro-organisms has important implications for vaccine development.

Among the humoral mechanisms of the innate immune system, the alternative pathway of complement activation is perhaps the most important one. While the classical pathway of complement activation requires the presence of specific antibodies (and hence is delayed upon microbial infection), the alternative pathway is initiated in the absence of antibodies. A C3b homologue, generated through spontaneous cleavage of C3 present in the plasma, can bind to bacterial surfaces. Factor B of the alternative complement pathway binds non-covalently to C3b and is cleaved by a serum protease factor D, to yield a larger fragment (Bb) and a smaller fragment (Ba). The lytic pathway is triggered through binding of properdin (also called factor P) to C3bBb complexes. In contrast to host cells, bacteria lack complement-controlling membrane proteins such as decay accelerating factor (DAF; CD55), homologous restriction factor (CD59), and membrane co-factor protein (CD46) (Liszewski *et al.*, 1996). Through the C3/C5 convertase activity of the C3bBb complex, further C3 molecules are cleaved, leading to the production of large amounts of C3b homologues, which bind to bacterial surfaces and initiate the lytic pathway. Moreover, C3b deposition on microbial surfaces promotes the microbial uptake via complement receptors. Finally, the complement breakdown products C4a and C5a are chemo-attractants for phagocytes, and are, therefore, termed anaphylatoxins. They induce phagocyte extravasation into foci of microbial implantation.

◆◆◆◆◆◆ THE ADAPTIVE IMMUNE SYSTEM

While the components of the innate immune system are appropriate as a first line of defence, the adaptive (or specific) immune system is activated if the invading micro-organism cannot be eliminated, or at least be neutralized, by the above-mentioned non-specific effector mechanisms. Two major features characterize the adaptive immune system. First, the immune response is antigen-specific; specificity is made possible through the use of clonally distributed antigen receptors, i.e. surface Ig on antibody-producing B lymphocytes (Reth, 1992) and T-cell receptors (TCR) on the surface of T lymphocytes (Moss *et al.*, 1992). Secondly, the specific immune system develops memory. This allows the rapid response of antigen-specific effector cells upon second encounter of the relevant antigen (Mackay, 1993; Zinkernagel *et al.*, 1996).

B cells recognize antigen in a fashion which is fundamentally different from that of T cells. The antibody expressed on the B-cell surface (and, later, secreted by the plasma cell) directly binds to native, soluble antigen. By virtue of their antibody production, B cells contribute to the humoral immune defence against extracellular pathogens and neutralize virions before they enter the host cell. In contrast, T cells recognize antigen only if

it is presented in the context of appropriate MHC molecules on the surface of antigen-presenting cells (APC). The surface-expressed TCR is non-covalently associated with the CD3 polypeptide complex, which mediates signal transduction upon TCR triggering, leading to cytokine gene transcription and T-cell activation (Moss *et al.*, 1992; Chan *et al.*, 1994; Cantrell, 1996).

Two well-defined and some less well-defined subpopulations of T cells are involved in the specific cell-mediated immune response against infectious agents. The expression of relevant cell-surface molecules allows their identification and phenotypic characterization by specific monoclonal antibodies. Moreover, monoclonal antibodies can be used to separate subpopulations of cells by fluorescence-activated cell sorting (FACS) or by using magnetic beads. Accordingly, the chapter by Scheffold and Radbruch (Section I) is devoted to a detailed description of these techniques. The dominant subsets of mature T cells are characterized by the reciprocal expression of CD4 and CD8 coreceptors. $CD4^+$ T cells recognize antigen in the context of MHC class II molecules; they produce cytokines required for efficient activation of leukocytes (notably B cells and macrophages), and are therefore termed helper cells (Th cells). In contrast, $CD8^+$ T cells recognize antigen in the context of MHC class I molecules; one of their major tasks is to lyse virus-infected target cells. Hence, they are termed cytotoxic T lymphocytes (CTL) (Janeway, 1992). The activation of T cells requires two signals. Signal one is mediated through the CD3/TCR molecular complex following antigen recognition. Signal two is a co-stimulatory signal that is delivered through receptor–ligand interactions (Mueller *et al.*, 1989). The major co-stimulatory molecules on antigen-presenting cells are B7.1 (CD80) and B7.2 (CD86). Both molecules can bind to CD28 on T cells, thereby exerting co-stimulation. In addition, CTLA4 (CD152) also binds to both ligands. The CD28/CD152 interaction with CD80/CD86 is complex. Recent evidence indicates that triggering of CD28 mediates co-stimulation, whereas binding to CD152 delivers an inhibitory signal (Mueller *et al.*, 1989; Linsley *et al.*, 1994; Walunas *et al.*, 1994; Krummel and Allison, 1995; Tivol *et al.*, 1995; Lenschow *et al.*, 1996). An overview of some important cell-surface molecules with relevance to anti-infective immunity is given in Table 1.

The vast majority of the $CD4^+$ and $CD8^+$ T cells express a TCR composed of α- and β-chain heterodimers (Bentley and Mariuzza, 1996). These T cells are termed 'conventional T cells'. During the last few years, the existence of additional T-cell subsets has been appreciated. These include $CD4^-CD8^-$ 'double-negative' (DN) T cells, which express either the αβ TCR or an alternative TCR composed of γ and δ chains. Furthermore, $CD8^+$ T cells expressing the αβ TCR restricted by MHC class I like molecules, as well as $CD4^+$ T cells co-expressing the αβ TCR and natural killer (NK) receptors (NK1) have been identified. Increasing evidence suggests that there is a well-orchestrated interplay between these subsets with a preponderance of any one of them, depending on the type of infection.

Table 1. Cell surface molecules with relevance to anti-infective immunity

Cell surface molecule	Function in anti-infective immunity
TCRαβ	MHC/peptide recognition by the major αβ T-cell population
TCRγδ	Ligand recognition by the minor γδ T-cell population
CD1	Presentation of lipids and glycolipids to DN αβ T cells
CD3	Marker of all T cells, signal transduction in T-cells
CD4	Co-receptor with specificity for MHC class II, marker molecule of Th cell
CD8	Co-receptor with specificity for MHC class I, marker molecule of CTL
CD14	Pattern-recognition receptor on macrophages which, for example, binds LPS from Gram-negative bacteria
CD40	Co-stimulatory molecule on B cells and antigen-presenting cells
CD154 (CD40L)	T-cell co-stimulation (ligand for CD40)
CD28	Co-stimulatory T-cell molecule (positive signal)
CD152 (CTLA-4)	Co-stimulatory T-cell molecule (negative signal)
CD80 (B7-1)	Ligand for CD28, CD152
CD86 (B7-2)	Ligand for CD28, CD152
CD95	Fas (Apo-1), a receptor which mediates an apoptosis signal

LPS, lipopolysaccharide.

Conventional T Lymphocytes

$CD4^+$ T cells expressing the αβ TCR recognize foreign peptides bound in the peptide-binding groove of MHC class II molecules. These peptides are generally derived from exogenous antigens (such as micro-organisms) that are taken up by phagocytosis or endocytosis. Immunogenic peptides of 13 or more amino acids in length are generated in endosomes, bound to MHC class II molecules, and transported to the cell surface (Germain and Margulies, 1993; Cresswell, 1994; Germain *et al.*, 1996). Presentation of antigen to $CD4^+$ T cells is restricted to APC that either constitutively express MHC class II antigens (monocytes/macrophages, dendritic cells, B cells), or can be induced to express MHC class II molecules (e.g. endothelial cells and activated human T cells).

There are two functionally distinct subpopulations of $CD4^+$ Th cells, i.e. Th1 and Th2 cells. These subsets are distinguished based on the characteristic spectrum of cytokines that they produce upon antigenic stimulation (Abbas *et al.*, 1996). Th1 cells are characterized by their secretion of IFN-γ and IL-2, whereas Th2 cells preferentially produce IL-4, IL-5 and IL-10. As these cytokines play characteristic but different roles in various

types of immune response, Th1 or Th2 $CD4^+$ T cells can dominate in a given situation, thus determining the outcome of infection. Th1 cells play an important role in the initiation of the cell-mediated immune response against intracellular pathogens, due to secretion of IFN-γ (which activates macrophages) and IL-2 (which activates CTL). In the mouse, IFN-γ also stimulates the production of the Ig subclasses (IgG2a, IgG3) that contribute to antimicrobial immunity by virtue of their complement fixing and opsonizing activities. On the other hand, Th2 cells produce cytokines (IL-4, IL-5) that control activation and differentiation of B cells into antibody-secreting cells. Th2 cells are thus important for the induction of humoral immune responses. IL-4 controls the immunoglobulin class switch to IgE, and hence plays a central role in the immune defence against helminths and in the regulation of the allergic response. Moreover, IL-4 stimulates the production of IgG subclasses (IgG1 in the mouse) that neutralize but do not opsonize antigens. The Th2 cytokine IL-5, together with transforming growth factor β (TGF-β) induces B cells to switch to IgA, the major Ig subclass involved in local immune responses. In addition, IL-5 contributes to the control of helminth infection by activating eosinophils. Figure 1 illustrates the major effector functions of Th1 and Th2 subsets and the cytokine-driven regulatory interactions (Powrie and Coffman, 1993; Seder and Paul, 1994; Mosman and Sad, 1996).

Th1 and Th2 cells differentiate from an undetermined Th0 precursor cell. The differentiation into one or the other functional subset is driven by cytokines that are produced by cells of the non-specific immune system early after infection. In this regard, the rapid production of IL-12 by monocytes/macrophages, and of IFN-γ by NK cells, drives the Th cell

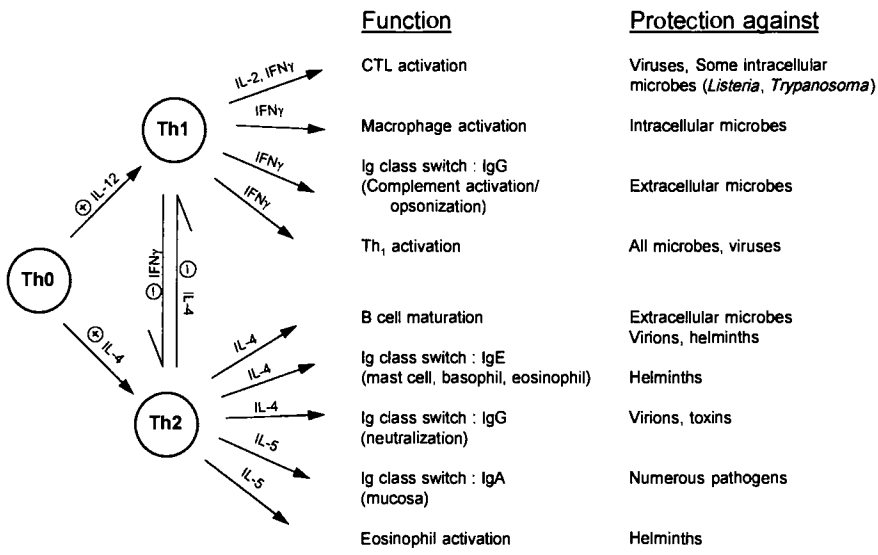

Figure 1. Role of Th1 and Th2 cells in anti-infective immunity. The differentiation from Th0 precursor cells is driven by IL-12 and IL-4, respectively. Th1 and Th2 cells are distinguished by a characteristic pattern of cytokine production. The key cytokines of Th1 and Th2 cells have differential roles in anti-infective immunity.

response into Th1 cells following bacterial infection (Seder and Paul, 1994; Lamont and Adorini, 1996). Conversely, the early production of IL-4 is a major force that drives Th cells along the Th2 differentiation pathway. The cellular source of the early IL-4 is not a classical T cell, but rather appears to include unconventional NK T cells as well as non-lymphoid cells such as basophils, eosinophils and mast cells (Seder and Paul, 1994; Bendelac *et al.*, 1997; Medzhitov and Janeway, 1997).

In most instances, there is no absolute restriction on the activation of either Th1 or Th2 cells during the immune response to infectious agents. Nevertheless, in many situations, there is a clear dominance of one or the other Th-cell subset, and a (genetic) failure to activate the appropriate Th-cell subset may lead to a disastrous outcome after infection. A well-documented example of this is the infection of mice with the protozoan parasite *Leishmania major*. Hence, technical aspects of working with the leishmaniasis model are discussed by Kropf and colleagues (Section II). In resistant strains of mice, such as C57Bl/6, *L. major* causes a self-healing lesion, whereas in susceptible Balb/c mice, the infection is progressive and eventually fatal. It has been shown that during infection, resistant mice produce high levels of IFN-γ and little IL-4 (and thus display a Th1-type response), whereas the susceptible mice produce high amounts of IL-4 and little IFN-γ (and thus display a Th2-type response) (Heinzel *et al.*, 1989). In view of the known role of IL-12 in driving Th0 cells into Th1 cells, attempts were made to prevent the fatal Th2 cell differentiation in *L. major* infected Balb/c mice. In fact, the administration of leishmanial antigens together with IL-12 induced the appearance of *L. major* specific Th1 cells in Balb/c mice. More importantly, these mice were protected from fatal infection when challenged with *L. major* (Heinzel *et al.*, 1993; Sypek *et al.*, 1993).

Similarly, the protective T-cell response against mycobacteria is mediated by Th1 cells. Mycobacteria induce IL-12 in macrophages, and IFN-γ secreted by Th1 cells is the major T-cell derived macrophage-activating mediator (D'Andrea *et al.*, 1992; Billiau, 1996). Tuberculosis is clearly dominated by a Th1 response; however, this may be insufficiently protective. The spectrum of disease observed in leprosy patients can be partially explained on the basis of a Th1 or Th2 preponderance. Whereas malign lepromatous leprosy is frequently associated with the production of Th2 cytokines, the more benign tuberculoid form of leprosy is dominated by Th1 cytokine patterns (Bloom *et al.*, 1992; Kaufmann and Andersen, 1997). Murine models of tuberculosis are described by Roberts and co-workers (Section II).

The concept of functionally distinct $CD4^+$ T cell subsets being differentially involved in immune responses on the basis of their cytokine production has greatly helped to delineate immune defence mechanisms in infection, and to devise therapeutic strategies. Attempts to identify surface markers that would allow unambiguous identification of Th1 and Th2 cells have not been too successful. It has been suggested that human Th1 and Th2 cells can be differentiated on the basis of their CD30 expression. According to this proposal, Th2 cells are $CD30^+$ while Th1 cells are $CD30^-$ (Del Prete *et al.*, 1995). However, there are well-documented cases where IFN-γ producing cells (thus being *bona fide* Th1 cells) were clearly

shown to express CD30 (Alzona *et al.*, 1995, Munk *et al.*, 1997). More recently, a differential expression of the β2 subunit of the IL-12 receptor on Th1 versus Th2 cells was reported. It was found that IFN-γ maintained the expression of IL-12R β2, and thereby the IL-12 responsiveness leading to Th1 differentiation, while IL-4 inhibited IL-12R β2 expression, leading to the loss of IL-12 signalling, associated with a differentiation into Th2 cells (Szabo *et al.*, 1997; Rogge *et al.*, 1997).

Differential expression of the IL-12R β2 subunit might turn out to be a useful marker for the discrimination of Th1 and Th2 cells, both in the murine and in the human system. Moreover, controlling the expression of the IL-12R β2 subunit could be an important target for therapeutic manipulation of developing Th-cell responses. It is obvious that a precise analysis of cytokine production is of major importance for the understanding of the pathophysiology of infection, as well as for designing rational strategies for therapeutic intervention. Therefore, several methods of measuring cytokines are described in this book. Depending on the experimental system, cytokine detection methods may include enzyme-linked immunosorbent assay (ELISA), bioassays, (semi)quantitative reverse transcription polymerase chain reaction (RT-PCR), or intracellular cytokine staining and FACS analysis (see the chapters by Scheffold and Radbruck (Section I), Fujihashi *et al.* (Section II) and Yssel and Cottrez (Section III)).

The prime task of $CD8^+$ CTL is the immune defence against intracellular pathogens. Viruses are replicated by host cells. As a consequence, viral proteins are degraded in the cytosol compartment of the cell. Virus-derived peptides are transported in association with the transporter of antigen processing (TAP) molecules into the endoplasmatic reticulum, where they are introduced to MHC class I molecules (Benham *et al.*, 1995; York and Rock, 1996). Upon transport to the cell surface, peptides of 8–10 amino acids in length are anchored in the peptide binding groove of MHC class I molecules, ready to be recognized by $CD8^+$ CTL expressing the appropriate TCR. While the important role of $CD8^+$ CTL in the elimination of virus-infected cells is well documented, there is clear evidence that MHC class I restricted $CD8^+$ T cells also contribute to the immune defence against intracellular bacteria (Kaufmann, 1993). Some intracellular microbes such as *Listeria monocytogenes* or *Trypanosoma cruzi* gain access to the cytosol, which causes their antigens to enter the MHC class I antigen processing pathway. In addition, it was found that bacteria-derived antigenic peptides can be introduced into the MHC class I pathway, despite the fact that the micro-organisms themselves remain in the phagosome (Jondal *et al.*, 1996; Reimann and Kaufmann, 1997). Taken together, there is a preponderance of $CD4^+$ T cells in the immune defence against phagosomal pathogens, and of $CD8^+$ T cells in the immune defence against cytosolic pathogens. In many instances, however, optimal protection against infectious agents requires the co-ordinated co-operation of $CD4^+$ and $CD8^+$ T cells.

$CD8^+$ CTL can eliminate infected cells, thereby limiting pathogen spread. Cytotoxicity is mediated through pore-forming proteins (perforins) and enzymes (granzymes) that are released by activated CTL upon

cell-contact-dependent recognition of relevant (e.g. virus-infected) target cells (Berke, 1994). Techniques to measure killer-cell activity in murine and human systems are described in the chapters by Busch and Pamer (Section II) and Pawelec *et al.* (Section III), respectively. CTL can also trigger programmed cell death (apoptosis) in target cells through receptor–ligand interactions (Takayama *et al.*, 1995). Upon activation, CTL are induced to express *Fas*-ligand (*Fas-L*), a member of the tumour necrosis factor α (TNFα) gene family. *Fas-L* interacts with the corresponding receptor *Fas* (CD95, APO-1) expressed on virus-infected target cells. The oligomerization of several *Fas* molecules triggers a rapid suicide programme, which culminates in protease-dependent cell death, usually associated with fragmentation of genomic DNA into oligonucleosomal-sized fragments (Nagata, 1994). However, increasing evidence indicates that the role of $CD8^+$ T cells in infection is not limited to their function as CTL. Like $CD4^+$ T cells, $CD8^+$ T cells are equipped with the capacity to produce cytokines. More specifically, the range of cytokines secreted by $CD8^+$ T cells depends on the antigenic stimulation, in a manner comparable to the situation with $CD4^+$ T cells. This has led to the suggestion that $CD8^+$ T cells should also be divided into subsets (Tc1, Tc2), based on their cytokine-secretion profile (Mosman and Sad, 1996).

The dominant role of T lymphocytes in the immune defence against infectious micro-organisms requires a broad spectrum of appropriate methods for analysis. Therefore, separate chapters are devoted to the isolation of lymphocytes from infected animals (Czuprynski and Brown, Section II), the establishment of T-cell lines and clones from murine (Born *et al.*, Section II) and human lymphocytes (Märker-Herrmann and Duchmann, Section III). Methods of immortalizing human T cells by herpersvirus saimiri are described by Fickenscher and Fleckenstein (Section III).

Unconventional T Lymphocytes

Apart from the well-characterized $CD4^+$ and $CD8^+$ T-cell subsets that recognize antigenic peptides in an MHC class II or MHC class I restricted manner via the conventional αβ TCR, several additional T-cell populations can contribute to the immune defence against infectious micro-organisms. These additional T-cell subsets have been collectively termed 'unconventional' T-cells (Kaufmann, 1996).

In mice, $CD8^+$ T cells have been described that express the conventional αβ TCR, and recognize an unusual group of peptides in the context of MHC class I-like presenting molecules (Lenz and Bevan, 1996). The peptides carry the *N*-formylmethionine (*N*-f-met) sequence that represents a characteristic signal sequence required for protein export in bacteria. *N*-f-met containing peptides are virtually absent from mammalian cells, with the exception of mitochondria. The MHC class I-like molecules that present *N*-f-met containing peptides to $CD8^+$ T cells are far less polymorphic than classical MHC antigens, and hence are broadly distributed (Shawar *et al.*, 1994). $CD8^+$ T cells with specificity for *N*-f-met containing peptides

have been shown to mediate protection in experimental murine models of *Listeria monocytogenes* infection (Lenz and Bevan, 1996). The possible role of such cells during the immune response in humans is less clear, because homologues of the relevant murine MHC class I-like molecules have so far not been identified in man.

Additional unconventional T-cell subsets exist that recognize antigen in association with non-MHC molecules. Among those, DN T cells have been identified in humans that recognize non-peptide antigens in the context of CD1 gene products, which share some similarities with MHC class I molecules. These T cells express the αβ TCR. Interestingly, these T cells can recognize lipoids derived from mycobacteria, including mycolic acid and lipoarabinomannan (Beckman *et al.*, 1994; Sieling *et al.*, 1995). There is only limited polymorphism of the presenting CD1 molecules. The group-1 CD1 molecules (CD1a–c) required for presentation of glycolipids to these DN T cells are expressed on the surface of human macrophages, or can be induced to be expressed on APC (Blumberg *et al.*, 1995; Porcelli, 1995). DN T cells with specificity for glycolipids have not been described in mice, possibly because the cognate of human group-1 CD1 antigens are absent in this species. The DN αβ T cells secrete IFN-γ and express cytolytic activity. Hence they could contibute to antimicrobial defence in a way similar to conventional Th cells.

Another subset of unconventional T cells is characterized by co-expression of αβ TCR and NK1, a characteristic marker of NK cells. These T cells recognize ligands in the context of group-2 CD1 molecules (CD1d). Interestingly, the TCR repertoire of NK1 T cells is strikingly restricted. The TCR of these T cells is composed of an invariant α chain associated with a β chain that uses a limited set of variable (Vβ) elements, suggesting that NK1 T cells can recognize only a restricted array of antigens. An interesting functional feature of these cells is the rapid induction of IL-4 production, indicating that NK1 T cells promote Th2 cell differentiation (MacDonald, 1995; Bendelac *et al.*, 1997).

While the vast majority of $CD3^+$ T cells express the 'conventional' TCR composed of an αβ chain heterodimer, a minor subset (1–10%) of $CD3^+$ T cells expresses the alternative γδ TCR (Porcelli *et al.*, 1991). There are two major differences between αβ T cells and γδ T cells. First, the majority of γδ T cells lack the expression of co-receptor molecules CD4 or CD8, thus displaying a DN phenotype. Secondly, the number of germ-line gene elements that can be expressed to construct the variable regions of TCR chains is small for γ and δ as compared with α and β (Porcelli *et al.*, 1991; Haas *et al.*, 1993). Nevertheless, the available TCR repertoire of γδ T cells is at least as large as that of αβ T cells, because several non-germ-line encoded mechanisms such as N-region diversity, use of alternative reading frames, etc., dramatically contribute to TCR diversity. Substantial evidence suggests that γδ T cells play a role in the immune defence against various infectious micro-organisms (Haas *et al.*, 1993; Kaufmann, 1996). Human γδ T cells expressing the Vγ9/Vδ2 TCR are strongly activated by live or killed mycobacteria, as well as by several other intracellular or extracellular bacteria, or protozoa such as *Plasmodium falciparum* (Kabelitz, 1992; Haas *et al.*, 1993). In several instances, a transient

increase in circulating γδ T cells has been observed during acute infection. γδ T cells express a functional repertoire similar to conventional αβ T cells. Thus, activated γδ T cells exert CTL activity and produce a range of cytokines, depending on the antigenic stimulation (Kabelitz, 1992; Haas *et al.*, 1993). A Th1 pattern of cytokines was produced by peritoneal γδ T cells when mice were infected with *Listeria monocytogenes*, whereas peritoneal γδ T cells produced Th2-type cytokines when mice were infected with *Nippostrongylus brasiliensis* (Ferrick *et al.*, 1995). The microbial ligands recognized by human Vγ9/Vδ2 T cells have recently been characterized as non-proteinaceous, phosphate containing low-molecular-weight compounds. These ligands include phosphorylated nucleotide-containing compounds termed TUBag (Constant *et al.*, 1994) as well as prenylpyrophosphates such as isopentenylpyrophosphate (IPP) and related compounds (Tanaka *et al.*, 1995). The recognition of these ligands by human γδ T cells is not restricted by classical MHC antigens or other presenting molecules (such as CD1), but requires some as yet ill-defined form of presentation. While the microbial phospholigands are potent activators of human γδ T cells, they do not appear to be recognized by their murine counterparts. Instead, there is evidence that some murine γδ T cells recognize heat shock proteins (hsp) derived from micro-organisms such as *M. tuberculosis* (Born *et al.*, 1990; Kaufmann, 1996).

Despite their impressive *in vitro* reactivity towards certain ligands from infectious micro-organisms, the *in vivo* role of γδ T cells in infection is not precisely understood. In several experimental models of bacterial infection, a transient activation of γδ T cells during early phases of the immune response is observed. On the other hand, γδ T cells appear to contribute to protection against certain viral infections at later stages. In this context, it is interesting to note that characteristic changes in the expressed TCR repertoire of peripheral blood γδ T cells occur in individuals infected with the human immunodeficiency virus (HIV) (Hinz *et al.*, 1994). A protective role of γδ T cells in experimental tuberculosis was revealed through the analysis of gene deletion mutant mice deficient in γδ T cells. It was found that these $C\delta^{-/-}$ knock-out mice succumb to lethal infection with *M. tuberculosis*, but only if high inocula of *M. tuberculosis* are used (Ladel *et al.*, 1995; D'Souza *et al.*, 1997).

Although γδ T cells comprise only a minor subpopulation of peripheral T lymphocytes, they may play a decisive role in the immune defence against certain micro-organisms. Therefore, this aspect needs to be considered in the development of new subunit vaccines, e.g. against tuberculosis. In addition to well-defined protective αβ T-cell epitopes, such subunit vaccines might include non-proteinaceous phospholigands stimulating γδ T cells. On the basis of the rapid response of γδ T cells (frequently preceding that of αβ T cells) and their limited germ-line TCR repertoire, it is assumed that γδ T cells provide a link between the innate and the adaptive immune system (Boismenu and Havran, 1997). In addition, a more general regulatory role of γδ T cells in inflammation appears likely.

B Lymphocytes

B cells express surface Ig as their antigen-specific receptor molecules. Upon activation and differentiation into antibody-secreting cells, B cells produce and secrete large amounts of Ig with the same specificity as the membrane-bound Ig. T-cell-dependent B cell activation requires cognate interaction between the two lymphocyte populations. In recent years, it has become obvious that the receptor–ligand interaction mediated between CD40 (expressed on B cells) and the corresponding receptor expressed on T cells (CD40 ligand or gp39; now termed CD154) is important for the initiation of humoral immune responses to T-cell-dependent antigens (Foy *et al.*, 1996). In addition, studies with gene deletion mutant mice lacking either CD40 or CD154 expression have shown that CD40/CD154 interactions are essential for secondary immune responses to T-cell-dependent antigens, as well as for the formation of germinal centres (Grewal and Flavell, 1996).

Proliferation and differentiation of B cells as well as the Ig isotype class switching are driven by cytokines (Stavnezer, 1996). In the mouse, IL-4 induces IgG1 and IgE secretion, while TGF-β and IL-5 trigger the IgA class switch. IFN-γ is known to preferentially induce IgG2a and IgG3 secretion. IgG3 (together with IgM) possesses complement-fixing activity. These Ig subclasses are thus involved in the initiation of the classical pathway of complement activation, leading to complement-mediated destruction of pathogens or infected cells. In addition, antibodies are required for antibody-dependent cellular cytotoxicity (ADCC) effector function. Lymphoid cells carrying receptors for the Fc portion of IgG (Fcγ receptor), such as large granular lymphocytes, mediate ADCC of IgG-coated target cells.

The initial encounter of antigen-specific B cells with the appropriate Th cells occurs at the border of T and B cell areas in lymphoid tissues. Activated B cells migrate into a nearby lymphoid follicle where they form a germinal centre. In the germinal centres, somatic hypermutation occurs in rapidly proliferating B cell blasts, thus giving rise to the selection of high-affinity antibodies (affinity maturation) (Rajewsky, 1996).

In addition to their unique role as antibody-producing plasma cells, B cells have the capacity to present antigen to T lymphocytes. Upon binding of soluble antigen to membrane-bound Ig with homologous specificity, antigen–antibody complexes are internalized and degraded in the endolysosomal compartment. Antigen-derived peptides are then introduced to the MHC class II-dependent processing pathway and can be presented to appropriate peptide-specific $CD4^+$ T cells (Watts, 1997).

◆◆◆◆◆ CYTOKINES

Collectively, cytokines are soluble mediators that exert pleiotropic effects on cells of the immune system and transduce signals via specific surface receptors (see Table 2). Cytokines primarily produced by cells of the

Table 2. Cytokines with relevance for the anti-infective immune response

Cytokine	Major role in antimicrobial defence
Chemokines	Leukocyte attraction to site of microbial implantation
CXC chemokine	Granulocyte recruitment to site of microbial implantation
CC chemokine	Monocyte recruitment to site of microbial implantation
C chemokine	Lymphocyte recruitment to site of microbial implantation
IL-1	Proinflammatory, endogenous pyrogen
IL-6	Proinflammatory
TNF-α	Proinflammatory, macrophage co-stimulator, cachexia
IL-2	T-cell activation
IFN-γ	Macrophage activation, promotion of Th1 cells
IL-4	B-cell activation, switch to IgE, promotion of Th2 cells, activation of mast cells
IL-5	Switch to IgA, activation of eosinophils
IL-12	Promotion of Th1 cells
IL-10	Anti-inflammatory
TGF-β	Anti-inflammatory

immune system with known cDNA sequence are designated interleukins. As discussed above, Th cells are functionally differentiated into Th1 and Th2 subsets on the basis of their characteristic cytokine spectrum (Abbas *et al.*, 1996). Upon appropriate activation, these Th subsets produce interleukins that are primarily required for immunological control of intracellular pathogens (Th1) or the regulation of Ig class switching (Th2). In addition, cytokines produced by monocytes and macrophages (frequently termed monokines) have important roles in the immune defence against infectious agents. Cytokines produced by macrophages in response to stimulation with bacterial components include IL-1, IL-6, IL-12 and TNFα.

IL-12 is a driving force for the differentiation of Th1 cells from undetermined Th0 precursor cells (Trinchieri, 1995). IL-1 (Dinarello, 1992), IL-6 (Akira *et al.*, 1993) and TNFα (Tracey and Cerami, 1993) are proinflammatory and pleiotropic cytokines that induce a variety of effects on many different target cells. A large group of cytokines is collectively termed 'chemokines'. These proteins recruit phagocytic cells and lymphocytes to local sites of infection. Chemokines are characterized by four conserved cysteines forming two disulphide bridges. The position of the first two cysteines has been used to divide the cytokines into two families: the C-X-C (or α) and C-C (or β) chemokines (Baggiolini *et al.*, 1997). IL-8 and NAP-2 are members of the C-X-C chemokine family that promote the

migration of neutrophils. MIP-1β, MCP-1 and RANTES are members of the C-C family of chemokines that promote migration primarily of monocytes and T lymphocytes (Mackay, 1996). Chemokines can be produced by many different cell types in response to stimulation with bacterial antigens or viruses. As a consequence, local recruitment of phagocytic and effector cells due to the effect of chemokine release is a general feature of the immune response to infection. In addition, it has recently been discovered that chemokines and their receptors play important roles in the control of HIV infection of target cells (D'Souza and Harden, 1996).

Several cytokines possess anti-inflammatory activity. IL-10, produced by monocytes and Th2 cells, inhibits synthesis of IL-12 and Th1 cytokines IL-2 and IFN-γ, thereby supporting the differentiation of Th2 cells, which is mostly promoted by IL-4 (Moore *et al.*, 1993). Similarly, TGF-β is a potent inhibitor of macrophage and lymphocyte activation (Wahl, 1994). These cytokines contribute to the termination of ongoing inflammatory responses.

◆◆◆◆◆◆ CONCLUDING REMARKS

As briefly summarized in this chapter, the immune response to infectious agents involves a broad spectrum of mechanisms of the innate and the acquired immune systems. Accordingly, the analysis of these mechanisms requires a similarly broad spectrum of sophisticated immunological methods. While some chapters of this book describe methods that are generally appplicable to the analysis of murine and human immune responses, other chapters deal with methods that are particularly useful for the analysis of specific infection models, such as tuberculosis or leishmaniasis. Although the analysis of specific problems might require additional methods, we believe that this book provides a useful guide for the microbiologist wishing to use immunological methods to investigate infection and infectious disease.

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PART I

General Methods

1 Phenotyping and Separation of Leukocyte Populations Based on Affinity Labelling
2 Measurement of Cellular Proliferation
3 Prediction and Determination of MHC Ligands and T-cell Epitopes

1 Phenotyping and Separation of Leukocyte Populations Based on Affinity Labelling

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CONTENTS

Introduction
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INTRODUCTION

The combination of monoclonal antibody technology with flow cytometry provides a powerful tool for detailed molecular phenotyping and isolation of individual cells according to the expression of specific proteins at resolutions down to single amino acid differences in protein sequence. Specific fluorescent or magnetic labelling comprises not only antibodies but can also be extended to all kinds of specific high-affinity ligand–receptor interactions, and we will therefore replace the term 'immunolabelling' by the more general term 'affinity labelling'. We will only focus on techniques that allow quantitative labelling, i.e. labelling proportional to 'antigen' density. Quantitative labelling can be achieved with free ligands or ligands conjugated to colloidal magnetic particles (MACS System (Miltenyi *et al.*, 1990)) or liposomes, and will here be termed 'staining'. In contrast, larger particles in the micrometer range will allow only qualitative labelling ('all or nothing'). Cytometry, i.e. affinity-based phenotyping in its traditional and newer forms, which will be introduced here, allows specific quantification of nucleic acids, intracellular, surface and secreted proteins, hormones and sugars. This is the analytical basis for the preparative approaches of fluorescence-activated cell sorting (FACS) and high-gradient magnetic cell separation (MACS), which allow isolation of subpopulations and individual cells of defined phenotype down to frequencies of less than one in 10^7 cells (Radbruch and Recktenwald, 1995).

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◆◆◆◆◆◆ AFFINITY-BASED FLUORESCENT LABELLING

Basic Considerations

Available parameters

Light scatter

Cytometry allows analysis of single cells according to light scattering and emission of fluorescent light. State-of-the-art flow cytometers (such as Becton-Dickinson's FACS series and Coulter's ELITEs) detect light scatter at an angle of 2–20° (forward scatter (FSC)) and 90° (side scatter (SSC)) relative to the axis of the illumination. Light scatter gives information about cell size (FSC) and granularity (SSC), which allows identification of various leukocyte subpopulations such as monocytes, granulocytes, lymphocytes, cellular debris and cell aggregates.

Fluorescence

Apart from scattered light, flow cytometers detect fluorescent light, emitted from fluorochromes upon excitation by the illuminating light. The number of parameters (colours) is restricted by the number of available dyes, which must fulfil the following criteria:

- they must be excitable by the illuminating light source (usually a 488 nm argon laser)
- they should have high quantum yield (ratio of absorbed to emitted photons)
- their fluorescence emission must be distinguishable from that of the other dyes used
- it should be possible to conjugate them to proteins.

Although research cytometers exist that use helium–neon, dye or diode lasers and even mercury lamps as a light source, we will focus here on the standard combination of three different dyes and an argon laser as the light source.

Fluorescein (FL; excitation maximum Ex_{max} 495 nm, maximum of emission (Em_{max}) 520 nm), a dye which can easily be conjugated to proteins, is commonly used together with the algal phycobiliprotein phycoerythrin (PE; Ex_{max} 480, 545, 565 nm, Em_{max} 575 nm). PE displays brighter fluorescence than FL, due to its higher absorption and quantum yield. Another phycobiliprotein, peridinin chlorophyll-*a* (PerCP; Ex_{max} 470 nm, Em_{max} 680 nm), or tandem conjugates of PE with Cy5 (Ex_{max} 650 nm, Em_{max} 666 nm) or related dyes can be used as a third colour. Energy-transfer systems such as PE/Cy5 absorb light via PE and the energy of the emitted light is directly 'transferred' to excite fluorescence emission of the second dye at a longer wavelength. This results in a wide separation of the excitation and emission wavelengths (Stokes' shift). An overview of standard dyes and their possible combinations for four-colour measurement using a single argon laser (Coulter ELITE) or an additional red diode laser (recently introduced in Becton-Dickinson's FACSCalibur) is given in Table 1.

Table 1. The most common dyes used in flow cytometry for three- or four-colour analysis.

Dye	Abbreviation, commercial name	Ex_{max} (nm)	Em_{max} (nm)	Light source	Application
Fluorescein	FL	490	530	488 nm argon laser	Standard F1
Phycoerythrin	PE	480, 545, 565	575	488 nm argon laser	Standard F2
Cy5		650	666	630 nm red diode laser	F4 for four colour fluorescence (dual laser)
Phycoerythrin/Cy5	CyChrome, Tricolor, Red 670	Like PE+Cy5	666	488 nm argon laser + 630 nm red diode laser	F3 for three-colour fluorescence, F4 for four-colour fluorescence with single argon laser, *not* with red diode laser
Phycoerythrin/ Sulforhodamine 101	PE/Texas Red, ECD	Like PE+596	625	488 nm argon laser	F3 for four-colour fluorescence single argon laser
Peridinin-chlorophyll-*a*	PerCP	470	680	488 nm argon laser	F3 for four-colour fluorescence with Cy5 or APC or APC/Cy7
Allophycocyanin	APC	650	660	630 nm red diode laser	F4 for four-colour fluorescence (dual laser)
Allophycocyanin/Cy7	APC/Cy7	650	800	630 nm red diode laser	F4 for four-colour fluorescence (dual laser)

APC, allophycocyanin; ECD, energy-coupled dye; F1–F4, fluorescence channels; FL, fluorescein; PE, phycoerythrein; PerCP, peridinin chlorophyll-*a*.
Dye combinations for four-colour analysis with 488 nm, single argon laser excitation (e.g. Coulter ELITE) or dual laser excitation with an additional 630 nm red diode laser (e.g. BD FACSCalibur).

Alternatively, the third or fourth colour is often used to identify dead cells according to staining with propidium iodide (PI; Ex_{max} 536 nm, Ex_{max} 617 nm) for their exclusion from analysis. PI enters dead cells via their damaged cell membrane and intercalates into DNA, but is excluded from intact cells. PI and other DNA stains can conveniently be used to correlate the phenotype and the cell cycle stage (proliferation or apoptosis) (Darzynkiewicz and Crissman, 1990; Ormerod, 1994).

The third colour can also be used to identify nucleated cells by staining with LDS 751 (see protocol section), a vital DNA dye emitting in the far red, e.g. for optical separation of leukocytes in the presence of an excess of non-nucleated cells or particles (erythrocytes, cellular debris). Other dyes are available for correlation of the phenotype and biochemical parameters such as the redox potential, pH or calcium influx (Rothe and Valet, 1995).

Detection limit

Several thousand surface molecules per cell are required for cytometric detection by conventional fluorochrome conjugates (see later). For analysis of molecules expressed in lower frequencies, such as cytokine receptors or surface cytokines (Assenmacher *et al.*, 1996), ligands can be conjugated to fluorescent particles, e.g. magnetofluorescent liposomes (see later), which can increase signal to noise ratios 100- to 1000-fold.

Cell surface molecules

The most frequent application of affinity fluorescence is staining of surface molecules. The best characterized ones are classified by the cluster of differentiation (CD) nomenclature (Schlossman *et al.*, 1995). Surface molecules, which include structural, transport and communication molecules, already provide detailed information about the functional and differentiation status of a cell. They can be stained on live and on fixed cells, depending on the fixation protocol, and can be used to isolate live cells by affinity-label-based cell sorting with MACS or FACS.

Intracellular molecules

Many molecules are only present within the cell and not on its surface. As currently available affinity labels, such as antibodies, cannot penetrate intact cell membranes, intracellular molecules are not accessible in live cells. Intracellular molecules of interest include molecules involved in signal transduction, gene regulation, biochemical processes and also molecules *en route* to secretion or surface display. For intracellular staining cells have to be fixed, preserving their structural integrity, and membranes have to be permeabilized, allowing the affinity labels to reach the intracellular space (see later). In particular, for the analysis of heterogeneously and transiently expressed molecules, such as cytokines, intracellular affinity labelling and cytometry is the best technology available. The technique allows expression to be quantified at the level of individual cells, the kinetics of expression and frequency of expressing cells to be

determined in correlation to the surface phenotype and expression of other intracellular markers such as other cytokines (Assenmacher, 1992).

Secreted molecules

Cells can be analysed and sorted according to the expression of a defined secreted molecule using the cellular affinity matrix technology (see later) (Manz *et al.*, 1995). The basic approach resembles a cell-bound enzyme-linked immunosorbent assay (ELISA). An artificial affinity matrix is created on the surface of all cells by fixing an antibody or ligand to the secreted molecule on the cell membrane. This procedure does not affect cell viability and the labelled cells can be assayed for secretion *in vitro*. Upon secretion, secreted molecules bind to the affinity matrix on the surface of secreting cells. Diffusion of secreted molecules to non-secreting cells can be prevented by using a medium of high viscosity, low cell density or short secretion time. The secreted molecules can be stained in a similar way to conventional surface antigens, using a second antibody or ligand that recognizes a different epitope.

Sensitivity

The sensitivity of affinity fluorescence depends on the physical constraints of the cytometric hardware, which is discussed in detail elsewhere (Shapiro, 1988; Melamed *et al.*, 1990). The fluorescence phenomenon can be used for very sensitive detection, as the wavelength of the emission is increased compared to the excitation beam, which allows the background to be eliminated by optical filters (fluorescence is measured perpendicular to the excitation beam, where scattered light intensity is minimal). Further reduction of background is achieved by setting a trigger level, usually the FSC, excluding all events that do not reach a certain threshold.

Light is detected by photomultiplier tubes (PMTs), which are able to detect single photons. The light signal can be amplified either linearly, if little intensity variation is expected (e.g. DNA content), or logarithmically, for a higher dynamic range, which is usually the case for affinity labelling of cells, where differences of up to 1000-fold occur routinely.

The overall sensitivity of the instrument can be defined as the minimum number of dye molecules sufficient to separate two populations of standard beads (in general, around 500 FL molecules). These particles bear defined numbers of FL equivalents and show minimal variation in signal intensity (coefficient of variation (CV)).

The sensitivity of the measurement is also influenced by the nature of the sample. Fluorescence is a general property of many organic compounds, mostly aromatic or polyunsaturated molecules, which are present to varying degrees in all cells and generate 'cellular autofluorescence'. Autofluorescence correlates with cell size and cell characteristics, e.g. phagocytic activity. To be readily detectable the intensity of affinity staining must reach at least the order of magnitude of the

autofluorescence. The sensitivity of affinity labelling is strongly influenced by the staining reagents. The number of dye molecules that can be applied to a single cellular epitope increases in the order: direct reagents < indirect reagents < fluorescent particles (e.g. magnetofluorescent liposomes) (see later).

Quantification

Ligand number

In flow cytometry, the fluorescent label of each cell is measured quantitatively over a large range, usually 4 decades. Due to the sensitivity problems described above, the intensity is given as a relative, rather than an absolute, value. Quantitative statements require previous standardization. Standardization with unstained cells as reference allows easy determination of relative fluorescence intensities. To estimate the absolute numbers of stained molecules, the protein–fluorochrome conjugation rate of the staining reagent must be known, and the relative fluorescence intensity must be calibrated to an absolute standard (i.e. calibration particles with known numbers of fluorochromes). However, the information on conjugation rates may be limited, and conjugation may also change the fluorescence efficiency of the dyes and the antigen-binding properties of the conjugated ligands in an unpredictable way. To overcome these problems, calibration beads are used that have a defined binding capacity for the protein–fluorochrome conjugate. The fluorescence intensity of such beads and cells, both of which are stained under saturating conditions to eliminate affinity and valency differences, correlates directly to the number of surface antigens. Quantification kits using this type of particles are available from various suppliers (e.g. Quantum Simply Cellular, Sigma, St Louis, MI, USA).

Cell frequency and cell count

The frequency of stained subpopulations, as identified according to staining and scatter, are easily obtained by standard statistical analysis (see later). The high cell number analysable by flow cytometry is indeed one of the best ways to determine efficiently the frequencies of rare cells (Radbruch and Recktenwald, 1995). As the sample volume is not a fixed parameter in flow-cytometric analysis, absolute cell numbers have to be determined by adding a defined number of easily identifiable calibration beads to the defined total sample volume. The relative frequencies of the beads and the cells can then be used to calculate the cell number from the known bead concentration in the sample (e.g. TRUCOUNT, Becton Dickinson, San Jose, CA, USA).

Staining Reagents

Affinity labelling can be achieved in two ways: by using affinity ligands to which a fluorescent dye is covalently conjugated (direct staining), or by fluorescent reagents that stain specifically the primary affinity ligand

(indirect staining). Such 'secondary' reagents can recognize haptens, such as biotin, digoxigenin or nitrophenyl groups, which can easily be conjugated to proteins, and are detectable by avidin/streptavidin or specific antibodies. Frequently, antibodies are used that are directed against the heavy- or light-chain constant regions of the first labelling antibody, and which are iso-, allo- or xenotype specific. Liposomal or colloidal magnetic particles conjugated to direct or indirect affinity ligands differ in various aspects from molecular staining reagents and are discussed separately.

Direct staining

Direct staining is the preferred method for routine analyses and commonly used antigens. It requires minimal manipulation of the cells, which is important in maintaining cellular viability and minimizing cell loss. It is easy to control, and it allows labelling of several markers in one step. Many antibodies conjugated to standard fluorochromes are commercially available from a variety of suppliers, or can easily be prepared from purified proteins (see later). Staining is controlled by antibodies of the same xeno- and isotype, but irrelevant specificity, conjugated to the same fluorochrome at preferably the same rate (isotype control) and by staining cells that do not express the respective antigen (cellular control) (see later).

Indirect staining

Compared to direct staining, indirect staining may provide higher sensitivity because several secondary antibodies can bind to one primary antibody, thereby increasing the number of fluorochromes per target molecule. In addition, second-step reagents conjugated to exotic fluorescent dyes or protein dyes such as phycobiliproteins (PE, PerCP, allophycocyanin (APC), CyChrome) are commercially available and provide variability and convenience of multiparameter staining. Hapten systems are superior to allo, iso- or xenotype-specific antibodies in terms of specificity and convenience, and they are easy to control. For the most common hapten–ligand system, biotin–streptavidin, a vast array of conjugates is commercially available.

Allo-, iso-, or xenotype-specific antibodies may be used as secondary reagents for primary antibodies that cannot or have not been purified from culture supernatant, ascites or sera. Although sensitive, this method has some drawbacks with respect to multiparameter staining, specificity and cross-reactivity, especially if polyclonal antibodies are used. For multiparameter labelling, highly purified polyclonal or monoclonal antibodies are required. If the different primary antibodies happen to be of the same subclass, the various antigens must be stained sequentially with the primary and secondary antibody. Free binding sites of the secondary antibody must be blocked before adding the next primary antibody, and all staining and blocking steps must be controlled individually. These factors together make the entire procedure fairly tedious.

An additional problem of polyclonal as well as monoclonal anti-, iso-, allo-, or xenotype antibodies are low-affinity interactions with immunoglobulins other than the one of the main specificity, e.g. anti rat immunoglobulin G (IgG) may cross-react to mouse IgG on B cells. For polyclonal antibodies, this problem can be overcome by extensive absorption on cells expressing murine Ig (e.g. mouse spleen cells) or on affinity matrices (see later). For cross-reactive monoclonal antibodies this option does not exist, and such antibodies cannot be used.

Labelling with particles

Particles conjugated to affinity ligands show increased steric hindrance and reduced diffusion rates depending on their size, which may lead to prolonged incubation times and non-quantitative labelling. Small particles, such as liposomes and colloidal magnetic particles, still allow quantitative, although not saturating, labelling with an only slightly increased staining time (see later).

Magnetic colloids

Magnetic colloids, as used for high-gradient magnetic cell sorting, are superparamagnetic dextran-coated particles of irregular shape and diameter 50–100 nm, which is 5- to 10-fold the diameter of an IgG antibody. They bind to cells slower than protein ligands and are used under non-saturating labelling conditions (staining time 15 min). The weak magnetic label is sufficient to retain the cells in high gradient magnetic fields. Remaining free epitopes allow fluorescent labelling for analysis of sorting. The bound particles do not interfere with cytometric evaluation or biological function.

A major drawback of magnetic cell sorting compared to fluorescence-activated sorting has been its restriction to one parameter. However, magnetic beads are now available that can be released enzymatically from the cell surface, enabling magnetic separation via several parameters. A magnetic two-parameter sort is described later in this chapter.

Magnetofluorescent liposomes

Magnetofluorescent liposomes of defined size (about 400 nm in diameter) conjugated to affinity ligands and filled with fluorochromes and magnetic particles can greatly enhance the sensitivity of fluorescent labelling and allow magnetic separation of the cells. The magnetic label is also useful for sizing liposomes, i.e. for removing small liposomes, which would interfere with efficient labelling (Scheffold *et al.*, 1995).

Staining of cells with liposomes requires prolonged staining times (30–60 min) and gentle agitation during staining.

Conjugation of fluorochromes and haptens to proteins

Basic principles

For convenient conjugation of small fluorochromes or haptens to proteins, activated forms of these molecules are required that react with primary amino groups of the protein to form stable covalent bonds. Succinimidyl esters of fluorochromes and haptens which react efficiently at pH 8.5 are commercially available. Whenever possible, haptens are used with an extra spacer arm of about six carbon atoms to reduce steric hindrance. Sometimes isothiocyanate (ITC) derivatives of fluorochromes are used, such as fluorescein ITC (FITC). This requires a pH greater than 9 which can be deleterious for the antibody. The protocols given below for conjugation of succinimidyl esters can also be used for isothiocyanates, using 0.1 M boric acid, 0.025 M sodium borate, 0.075 M sodium chloride, pH 9.5 (adjust by NaOH) as coupling buffer. Antibodies should only be exposed to high pH levels for as short a time as possible.

Relevant parameters

The relevant parameters are stability and concentration.

Stability. Succinimidyl esters are sensitive to hydrolysis and therefore should be stored dry in a desiccator at –20°C. Stored aliquots should be equilibrated to room temperature before opening. Alternatively, succinimidyl esters can be stored dissolved in *water-free* dimethyl sulphoxide (DMSO) at high concentration (5 mg ml^{-1}), frozen in aliquots for single use. Aqueous solutions are prepared immediately before conjugation and instantly added to the protein solution. All buffers must be free of strong nucleophils, such as amines (e.g. Tris buffer), azide or stabilizing proteins, which would interfere with the coupling reaction.

Concentrations. The conjugation ratio, i.e. the number of haptens or fluorochromes conjugated to a single protein molecule, depends on: (a) the absolute concentration of the reagents, and (b) the molar ratio of dye or hapten to protein. Protein concentrations are optimal at about 1 mg ml^{-1} and the dye concentration should be varied to determine the optimal ratio (for IgG, molar ratios of 10:1 to 50:1 usually give the best results). Low conjugation ratios (1–2) provide only low signal intensity, while too high ratios (> 10) result in non-specific hydrophobic interaction with cells, inactivation of the affinity reagent, or its precipitation. Precipitates can be removed by centrifugation (12 000*g*, 10 min).

Staining Parameters

The factors that influence immunofluorescent staining, e.g. the concentration of cells and reagents, time, temperature, buffer, cell type and viability, must be optimized to obtain optimal and reproducible results. In general, conditions should be chosen to give the brightest positive signal possible and minimal background staining. Cells should be treated gently, with as few as possible steps of manipulation.

Conjugation of fluorochrome- and hapten-succinimidyl esters to proteins

1. Transfer protein into conjugation buffer (phosphate buffered saline (PBS) or 0.1 M sodium carbonate pH 8.5) by dialysis or gel filtration on PD-10 (up to 2 ml) or NAP5 (up to 0.5 ml) columns (Pharmacia, Uppsala). Adjust concentration to about 1 mg ml^{-1}.
2. Dissolve succinimidyl esters in DMSO at 5 mg ml^{-1}. Water-soluble sulfo-reagents can be dissolved in buffer (precipitates may be removed by centrifugation: 12 000*g*, 1 min).
3. Immediately add the dissolved succinimidyl esters to the protein solution to obtain the appropriate molar ratio (e.g. for IgG start with a molar ratio of 20 : 1, see above) and mix.
4. After 1 h at room temperature, remove free fluorochromes or haptens by dialysis against PBS/0.05% sodium azide or by gel filtration.
5. The protein concentration and the average FL/protein molar ratio in the conjugate can be determined according to the optical density (OD) at 280 and 495 nm. Haptens such as biotin or digoxigenin display no specific light absorption, making determination of the conjugation ratio tedious. Such conjugates can only be tested functionally, e.g. by titration (see Fig. 1). For IgG (M_r 150 000):

$$\text{FL/protein} = 2.9 \times \frac{\text{OD } 495}{\text{OD } 280 - 0.35 \times \text{OD } 495}$$

$$= \frac{\text{OD } 280 - (0.35 \times \text{OD } 495)}{1.4} \text{ mg ml}^{-1}$$

6. Titrate conjugates on test cells as described below and freeze stock solution in small aliquots at –70°C. Do not freeze/thaw repeatedly.

Conjugation of phycobiliproteins to proteins

The controlled cross-linking of proteins and purification of conjugates will not be discussed. Details can be found elsewhere (Hermanson, 1996). An easy protocol is provided in Mueller (1992). Detailed conjugation protocols are provided via the internet (e.g. http://cmgm.stanford.edu/~roederer/abcon.html).

Volume

The concentration of cells in the staining volume should be chosen such that cell viability is not impaired and that the staining reaction can be adapted to variable cell numbers by simply changing the reaction volume. Neither the concentration of the label nor the concentration of cells should be changed. This provides standard conditions for the staining reaction. As a rule of thumb, up to 10^7 cells can be stained in 100 µl. 100 µl of a 5 µg ml^{-1} solution of antibody contains approximately 2×10^{12} antibody

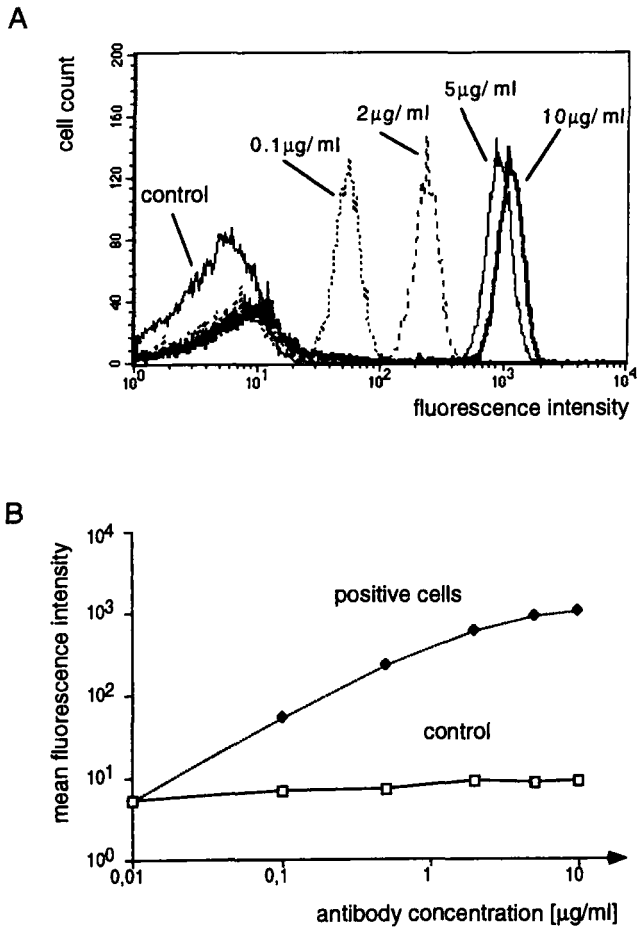

Figure 1. Titration of an anti CD4-PE conjugated antibody for staining human peripheral blood lymphocytes. The cells are stained with the concentrations indicated. (A) Plots of the fluorescence intensity versus the counted events; plots for all stainings are overlaid in one histogram. (B) Plots of the mean fluorescence intensities of the positive and negative populations versus the concentration of antibody; within the optimum range of concentrations (5–10 µg ml^{-1}) the staining is saturating, with minimum background.

molecules, which is sufficient to label 10^7 cells for antigens expressed at a frequency of 10^5 per cell. In principle, the staining volume must be adjusted to the number of positive cells and the antigen density per cell. For rare positive cells, however, the volume of the majority of negative cells will limit the volume of the staining. The lower limit of the number of cells for cytometric analysis is about 10^5 cells per sample, if 10^4 cells are to be analysed, because there is always a loss of cells due to washing and labelling. For routine analysis, 5×10^5 to 5×10^6 cells should be used. The staining volume should be 50–100 µl, but not less than 30 µl, otherwise the staining reagents cannot be added in precise concentrations. This has to be taken into account, especially when making quantitative comparisons of the labelling intensities of different samples.

Concentration of reagents

The concentration of the staining reagents has the strongest influence on the signal intensity versus the background labelling. Reagents should be used at optimal concentrations with the highest possible signal to noise ratio. Too low concentrations result in incomplete staining and low signal intensity, and thus yield suboptimal discrimination of positive and negative cells. Too high concentrations may result in unspecific staining, due to low-affinity cross-reaction of the labelling reagent. The optimum concentration of each reagent must be estimated by cytometric titration. Mixtures of positive and negative cells are stained under standard conditions, and labelling reagent concentrations, usually in the range 0.1–10 μg ml^{-1} are tested. The stained cells are analysed by flow cytometry and the optimum concentration is determined by plotting the mean fluorescence intensity (see later) of positive and negative cells versus the concentration of the labelling reagent (see Fig. 1).

Time

Specific binding of antibodies to cellular antigens occurs rapidly. Equilibrium is reached after 1-5 min, and 10 min is sufficient for most applications. Longer staining may lead to increased background, due to low-affinity cross-reactions which have slower kinetics. Longer staining times may be required if liposomes or magnetic beads are used as affinity labels (see below), and also for intracellular staining when the antibody has to penetrate the cellular membranes.

Buffer and temperature

In general, living cells are stained on ice, which reduces the physiological reactions of the cells, such as internalization, patching or capping and subsequent shedding of antigen. Apart from the temperature, sodium azide (0.02–0.05% in the staining buffer) can be used to block these physiological reactions reversibly.

The standard staining buffer is PBS, containing 0.5–1% bovine serum albumin (BSA) to saturate non-specific protein binding sites. To block non-specific binding, purified immunoglobulin (0.1–0.5 mg ml^{-1}) can be added to the buffer before or during the first staining step. Specific blocking antibodies for Fc receptors are also available.

Washing

During all staining steps cells should be handled carefully. Damaged and dead cells absorb staining reagents and release 'sticky' DNA, trapping viable cells and clogging nozzles of flow cytometers and MACS columns. Therefore it should be avoided to let the cells stand pelleted after centrifugation or to blow air through the cell suspensions. Cells should be kept on ice and cell pellets should be resuspended by gently flicking the

tube before adding new buffer, not by drawing the cells up and down in the pipette. Washing steps should be minimized in order to avoid cell loss (10% per washing step) and mechanical stress. One washing step, i.e. the addition of a 5- to 10-fold volume of washing buffer followed by centrifugation at 300*g* for 10 min, is sufficient to remove most unbound antibody. A second washing step will be necessary for indirect staining, where the primary antibody has to be removed completely, because otherwise it would react with the secondary reagent and reduce its available concentration.

Light

Most fluorescent dyes, especially phycobiliproteins, are sensitive to light. Absorption of light by the dye leads to the generation of reactive oxygen forms, which rapidly destroy dye molecules by oxidation. To prevent this 'photobleaching', antibody conjugates and stained cells should be protected from light.

Exclusion of dead cells

As dead cells impair staining, analysis and sorting, they should be removed, e.g. by centrifugation on a Ficoll density gradient, before the analysis. For analysis, they should be identified by the addition of the DNA dye propidium iodide (PI). This highly fluorescent red dye can penetrate the damaged cell membrane of dead cells and stain the DNA. Due to the fluorescence of PI, dead cells can be identified by being equally detected in fluorescence channels 2 and 3 (F2 and F3) (see later).

Fixation

Fixation of cells before analysis or staining is often used to limit the risk of infection and to standardize the time from staining to analysis. For intracellular stainings fixation is needed to stabilize the cellular structure. Subsequently, the cell membranes can be permeabilized for staining reagents by adding detergents. Standard fixation is done in 0.5–2% formaldehyde (for protein staining, see protocols below). For analysis of DNA or RNA, fixation in 70% methanol or ethanol/acetic acid (95/5 vol./vol.) is required. Most targets of labelling are not influenced by fixation, and labelling should be performed after fixation to minimize effects of fixation on the label. In some cases, fixation may not be advisable because it increases cellular autofluorescence. Also, some antigenic determinants (e.g. those containing lysine residues) are destroyed by reaction with aldehydes and can no longer be recognized by their ligand. This factor has to be tested for each antigen. Cells that were dead prior to fixation can be identified either due to scatter or by staining with LDS 751 (Terstappen *et al.*, 1988), which is not suitable for routine use. Apoptotic cells can be recognized by PI staining, since

their DNA staining is reduced, compared to $G_{0/1}$ cells, due to fragmentation of DNA.

Controls

Affinity labelling has to be controlled in terms of specificity. To this end, a labelling reagent as similar as possible to the reagent to be controlled, but with a different specificity, is used. For antibodies, isotype-matched antibodies are routinely used (isotype control). However, this control may be misleading, since different protein preparations and modifications may result in unpredictable differences. An alternative would be to stain cells that are similar to the target cells but do not express the target molecule (cellular control). The quality of this control depends on the similarity of the two cell types. Genetic mutants lacking the gene of interest would be ideal. In the case of transfectants, 'mock' transfected cells are the best control. Specificity also can be checked by blocking the staining with soluble target molecules, if available, or by preincubation of the target cells with unlabelled specific affinity reagent.

Data Acquisition and Analysis

Acquisition

During cytometric data acquisition, the five or six available parameters (FSC, SSC and three or four colours (F1–F4)) are all recorded for each particle that triggers measurement, i.e. for each particle that exceeds the preset threshold level for one of the parameters. Usually FSC is used as the trigger threshold.

Light scatter is analysed at linear amplification while fluorescence is measured logarithmically, to allow display of up to 10000-fold differences in intensity on one plot. Logarithmic amplification also emphasizes small differences in low numbers of antigens per cell, which often have high biological relevance because they may determine the functional phenotype of a cell (i.e. expression or not), rather than differences at high expression levels.

Apart from the trigger threshold, subsets of cells can be selected for analysis by setting upper and lower thresholds for any parameter. This 'live gating' may be necessary to obtain statistically significant numbers of rare cells for cytometric analysis. However, information about the excluded cells is invariably lost and unexpected or pathological situations may not be detected. Live gating should not be used in routine analyses. However, if it has to be done, an aliquot of cells should be analysed in parallel without a live gate for control purposes.

Compensation

Fluorescent light is emitted from a particular fluorochrome over a range of wavelengths characteristic for that dye. For dyes that have overlapping spectra (e.g. FL and PE), it is not always possible to measure each dye independently (Fig. 2). Spectral overlap can be corrected by compensation, i.e. subtraction of the relative contribution of the signal from the overlapping dye from the overall signal recorded in that parameter. Conventional flow cytometers have an electronic compensation circuit

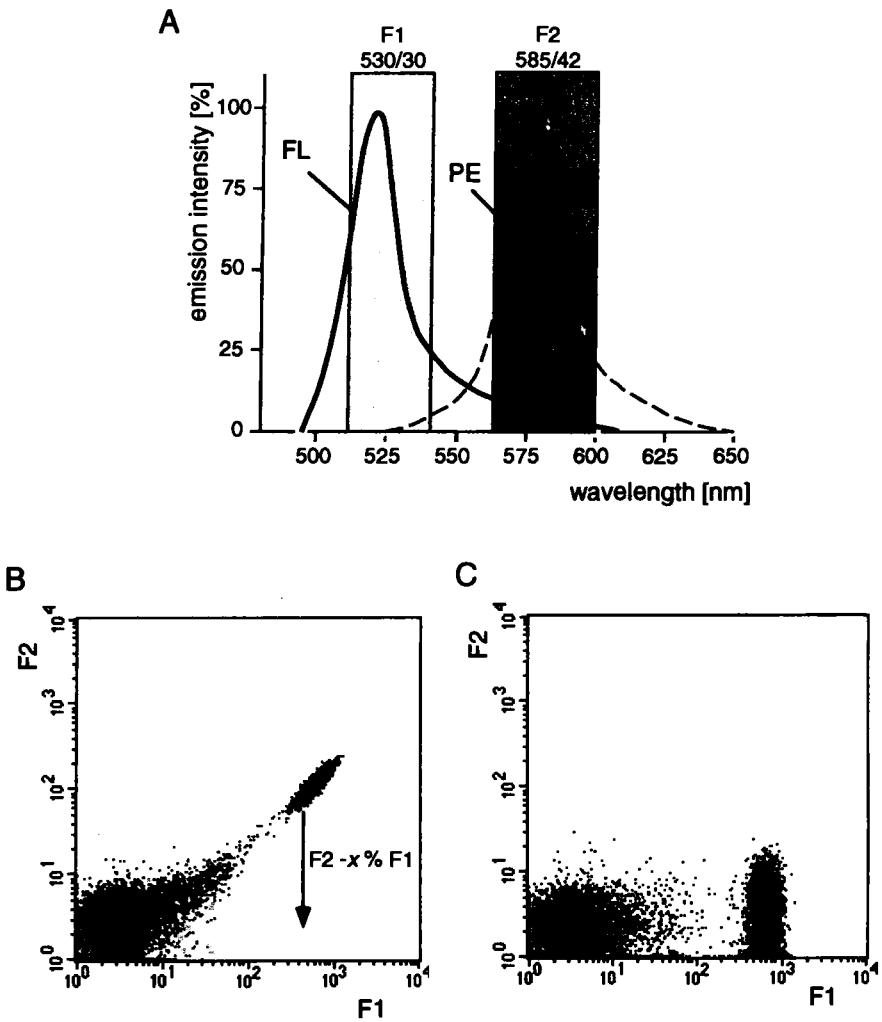

Figure 2. Spectral overlap and compensation. (A) The fluorescence emission spectra of fluorescein (FL) and phycoerythrin (PE), the bandwidths of the analysis filters (shaded bars). A certain percentage of the total fluorescence emission of fluorescein is detected in the F2 channel, and vice versa. (B, C) The effect of spectral overlap in a F1 versus F2 scatter plot for a single colour (F1) staining without (B) and with (C) electronic compensation of the F2 signal with x% of the measured F1 intensity subtracted from the F2 signal intensity.

which allows correction for spectral overlap, as illustrated by the following example.

The fluorescence intensity of FL, detected at 585 ± 21 nm (F2), is 10% of the fluorescence intensity detected at 530 ± 15 nm (F1). Therefore phycoerythrin fluorescence, detected at 585 ± 21 nm (F2), is electronically corrected by subtracting 10% of the F1 signal intensity of the same cell: F2(corrected) = F2 – 10% F1.

Compensation has to be established experimentally for each combination of dyes, by using a mixture of cells stained with the various dyes individually and at different degrees of brightness and adjusting compensation for all dyes. Standardized calibration particles are available for this purpose. Each dye must be compensated against all others. Some instruments may not have compensation for F1 versus F3 (>650 nm), and vice versa, as dyes are available that have no spectral overlap at these wavelengths. Calibration of compensation has to be done at an established PMT voltage. Changing the PMT voltage requires recalibration of the compensation. As the detection of fluorescence is not strictly linear over the whole range of four decades, compensation is best established with mixtures of dimly and brightly stained cells.

Data evaluation

Plotting and presentation of data

The usual way of plotting cytometric data is either as a one-parameter histogram or as a two-parameter dot plot or contour plot. One-parameter histograms emphasize the quantitative aspects of flow cytometry (relative intensities, population size, mean fluorescence, CV) for a single parameter. Two-parameter plots provide information on the correlation of expression of the antigens: in dot plots, each cell is indicated by a single dot, emphasizing small populations, while large populations show only poor resolution. Contour plots provide good resolution in areas of high cell density, as they delineate areas of equal cell density. Depending on the threshold limits, rare cells may not be shown.

In any case, graphical data presentation, including documentation of live and analysis gates, is preferred over just giving statistical results, such as the percentage of positive cells or the mean fluorescence, since differential gating and setting of statistical thresholds may cause significant differences in the final evaluation. This is particularly true in the case of weakly stained cells (see below).

Analysis gates

Live gates should be used with caution, since they preclude information from being recorded. Analysis gates are required for correct evaluation of the recorded data. They identify defined subpopulations of cells for statistical analysis. The current state of the art software still requires the operator to make subjective decisions about the definition of the gates. Gates can be verified either by using other parameters for control, or by

cell sorting for microscopic or functional analysis. Analytical gating is exemplified here for the analysis of white blood cells.

The first gate is set according to the light scatter of the cells. In the two-dimensional plot of forward versus side scatter lymphocytes, monocytes and granulocytes appear as separate populations distinct from small cells and debris (Fig. 3 (A)–(C)). According to the specific fluorescent labelling of lymphocytes, monocytes and granulocytes, gates can be defined for the forward versus side scattering of these populations. These forward versus side scatter gates will now serve as analytical gates for future analysis. Specific staining controls are extremely important in case of unexpected

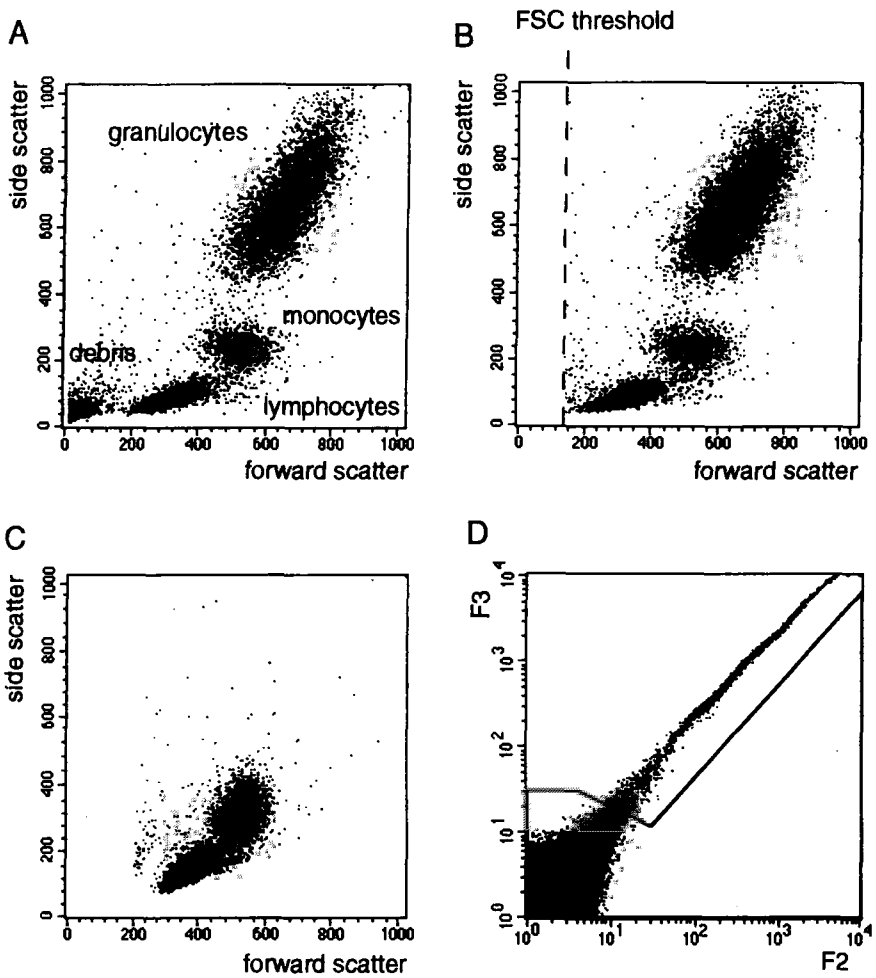

Figure 3. (A–C) FSC/SSC plots of peripheral blood leukocytes. Lymphocytes, monocytes and granulocytes can be identified by FSC/SSC properties. (A) After lysis of erythrocytes. (B) FSC threshold excludes debris from analysis. (C) Peripheral blood mononuclear cells; erythrocytes, granulocytes and dead cells are removed by centrifugation on a Ficoll gradient. (D) Exclusion of dead cells by PI staining. PI fluorescence is detected equally in the F2 and F3 channels, resulting in a diagonal of dead cells, which can easily be excluded from analysis by defining a gate in a F2/F3 scatter plot, as shown.

results, e.g. in pathological situations and for analysis of rare subpopulations. For example, activated cells frequently show increased light scatter. Dead cells are usually excluded due to their reduced forward scatter and to PI staining (Fig. 3 (D)).

Statistical analysis

Statistical evaluation of cytometrically defined populations mostly concerns the frequency of cells in a given population, their mean scatter or fluorescence intensity and the corresponding coefficient of variation (CV). The entire cell population has to be displayed 'on scale', since cells summed up in the first or last detector channel cannot be analysed for mean fluorescence or CV. The statistical mean is defined as the arithmetic or geometric mean for linear and logarithmic scales, respectively. Variations within a population are the result of biological variation (e.g. cell size and antigen density) and of analytical variation (e.g. stability of the intensity of the illuminating light, focusing and orientation of the cell during analysis). In logarithmic amplification a homogeneous population of cells results in a population that is distributed symmetrically about the mean value (Fig. 4). For such a distribution the CV is half of the peak width at 0.6 times the maximum height.

The intensity of the fluorescence is digitized into channels and can be converted to linear values for better quantitative comparison of the relative intensities of cell populations. The linear values L are calculated from the channel values C as

$$L = 10^{(C/X)}$$

where X is the number of channels per decade.

Populations separated entirely from each other in one or more parameters are easy to compare statistically: the statistical threshold can be

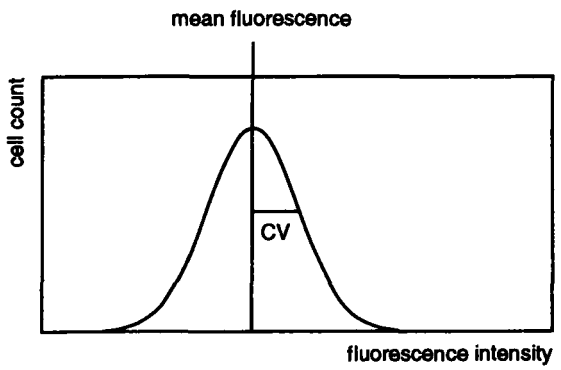

Figure 4. Fluorescence distribution of a homogeneous population of cells. Due to biological variations and variations in measurement, the cells are equally distributed around a mean value (mean fluorescence intensity (MFI)). The dispersion is described quantitatively by the coefficient of variation (CV). CV = SD/(MFI × 100), where SD is the standard deviation.

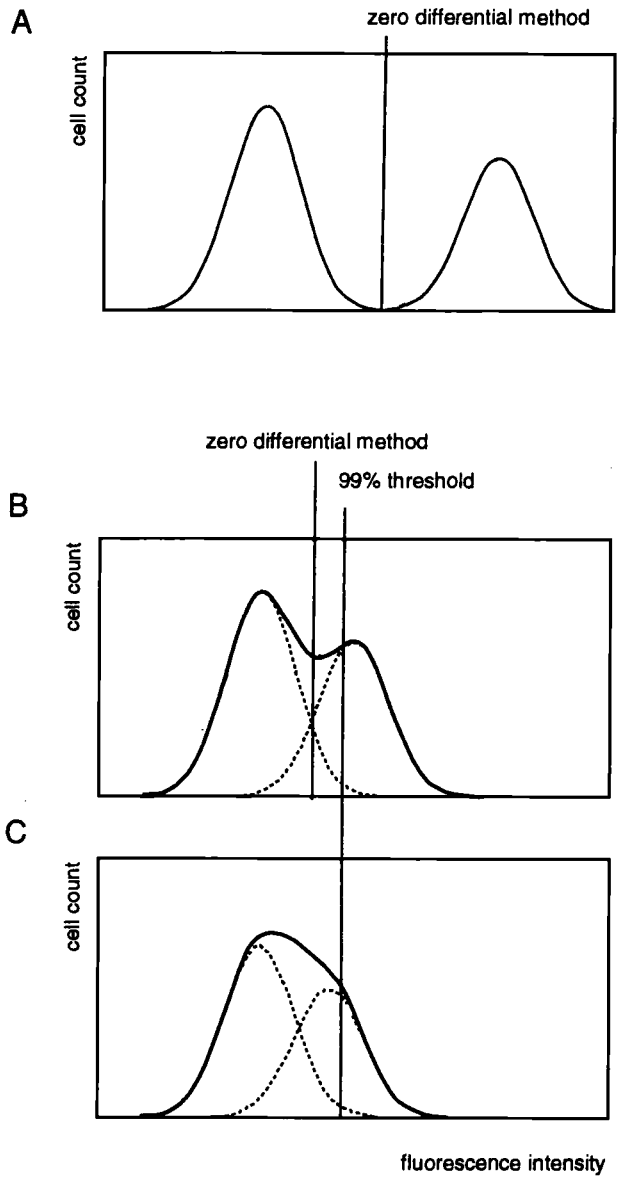

Figure 5. Statistical evaluation of separated (A) and bivariate (B) or asymmetrical (C) populations. (A) For separated populations analysis can easily be done by setting a statistical marker between the two populations (zero differential (valley) method), which can then be analysed separately for relative cell number, mean fluorescence intensity and coefficient of variation (CV). (B) Bivariate histograms require the use of curve-fitting programs, but for routine analyses they can be analysed using the valley method, assuming that the curve reflects two overlapping populations of similar size and CV so that false-positive and false-negative cells, defined by the marker, would cancel out. (C) Asymmetrical histograms, which may represent overlapping populations or kinetic transitions, allow only very preliminary analysis by standard evaluation methods, but require the use of curve-fitting programs. In all cases (A)–(C), the 99% threshold method provides no advantages, but rather leads to misinterpretation of the data.

set anywhere between the two populations (Fig. 5). If the two populations overlap, the statistical evaluation becomes more complicated. One method commonly used is to set a statistical threshold such that 99% of the negative control is included, and to regard all cells exceeding this threshold as positive for that parameter. This method cannot be recommended, as it will give results for separated populations that are incorrect by at least 1%, and completely wrong values for overlapping populations (see below). Also, for shifted populations, i.e. where there is an increase in mean fluorescence (compared to the control) but with a conserved CV, the 99% threshold method will give entirely incorrect results. As illustrated in Fig. 6, a weak shift in the average fluorescence of all cells, evaluated by 99% thresholding, will lead to a misinterpretation of the results.

For routine evaluation of overlapping populations, the threshold can be set at the lowest point between the two peaks (the 'zero differential', see Fig. 5). In the case of overlapping populations of about equal size, 'false-positive' and 'false-negative' cells will then level out. If populations differ too much in size (e.g. rare cell analysis) this method cannot be used. Ideally, statistical analysis of overlapping populations would involve the use of curve-fitting programs such as Modfit (Verity) or Multicycle (Partec). However, even these require information about the number of populations to be expected and to what extent kinetic differences may be involved. In any case it is preferable to improve staining conditions and to add additional discriminating parameters in order to achieve better resolution of the populations. For rare cells, enrichment prior to analysis may help identify the relevant population (Radbruch and Recktenwald, 1995).

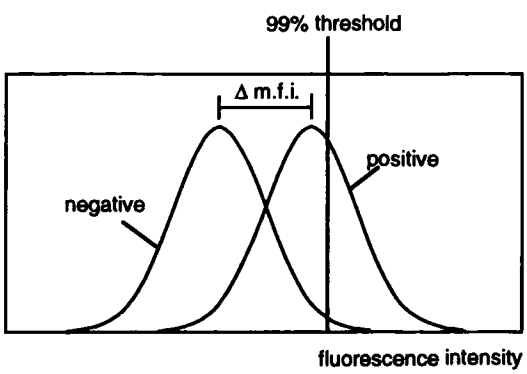

Figure 6. Shifted populations (histograms of stained and unstained cells are overlaid). All cells are stained without an increase in the coefficient of variation, and therefore setting statistical markers by any method would give false results. Such staining has to be described by the difference in the mean fluorescence intensity (Δ m.f.i.).

Standard Protocols: Staining and Instrumental Set-up

Sample preparation

For immunolabelling and flow-cytometric analysis, suspensions of single cells have to be prepared. Cell clumps must be removed by sedimentation and/or filtration, and buffers containing protein (BSA) and ethylene diaminetetraacetic acid (EDTA) should be used to reduce aggregation and non-specific staining. Details of how to obtain suspensions of single cells from various tissues are given in the chapter by Czuprynski and Brown in Section II.

Set up of a flow cytometer

The protocol given below can be used for standard flow cytometers with stable alignment. It serves to determine the instrument settings of threshold, gain and compensation for three-colour analysis of FL (F1), phycoerythrin (F2) and CyChrome (F3) fluorescence. Instead of cells stained for each colour separately, calibration particles (as described above) can be used. As the optical alignment is stable and settings can be stored and recalled, calibration must be performed only once for a particular type of analysis and a particular instrument. After changing the PMT voltage of any fluorescence parameter, the compensation must be set up again.

1. Choose logarithmic amplification for F1–F3 and linear amplification for FSC and SSC. Set all compensation levels to zero. Select FSC for threshold trigger and adjust the trigger level to about channel 50.
2. Run unstained cells and adjust the FSC/SSC until optimal separation of populations is obtained (with FSC around channel 300 for the smallest cells, e.g. lymphocytes). For leukocytes, see Fig. 3. Increase the trigger level to exclude debris, with all cells still clearly being above threshold.
3. Set a live gate around the population of the smallest cells (e.g. lymphocytes), with the lowest level of autofluorescence.
4. Adjust the PMT voltage of F1–F3 to yield a mean fluorescence intensity (MFI) of 3–4 (channel 120) for unstained cells. Some software allows the display of live statistics simultaneously during acquisition. The entire population should be 'on scale' (see above).
5. Run a mixture of unstained and FL-stained cells and display the results as a plot of F1 versus F2. Reduce the F2 signal of FL-stained cells by increasing F2 – (x% F1) until the MFI of F2 of the stained cells has reached the F2 value of the unstained population (MFI 3–4).
6. Use mixtures of unstained and PE-stained cells to adjust F1 – (x% F2) and F3 – (x% F2) (display as an F2 versus F3 dot plot), and mixtures of unstained and CyChrome-stained cells to adjust F2 – (x% F3) by repeating the procedure.
7. Run a mixture of all four populations. Cells will now be 'positive' for one colour only, having an MFI of around 3–4 for all other colours. Record 10^4 cells as a master file, which serves to document the calibration and as a source file for the instrument settings, to be recalled for future acquisitions.

Conventional surface staining

Direct reagents

1. Wash cells once with PBS/0.5% BSA/0.02% NaN_3 (PBA) (10^6 cells ml^{-1} 300*g*).
2. Gently flick the cell pellet and add 50–100 µl PBA containing titrated amounts of fluorochrome-conjugated antibodies (up to three different colours, each of which has to be controlled separately).
3. Incubate for 10 min on ice and protect from light.
4. Wash with 1 ml PBA.
5. Take up cells in about 0.2–1 ml of PBA for immediate analysis, or fix cells by adding 0.5% paraformaldehyde in PBS to the flicked pellet and store at 4°C in the dark until analysis.

Indirect staining using haptenized primary labels

1. Wash cells once with PBA (10^6 cells ml^{-1}, 300*g*).
2. Resuspend with 50–100 µl of haptenized antibodies in PBA (10 min, on ice).
3. Wash twice with 1 ml PBA.
4. Incubate with 50–100 µl of fluorochrome-labelled anti-hapten reagents (10 min, on ice).
5. Wash with 1 ml PBA.
6. Take up cells in about 0.2–1 ml PBA for immediate analysis, or fix as described above.

Indirect staining using isotype-specific reagents

1. Wash cells once with PBA (10^6 cells ml^{-1}, 300*g*).
2. Stain with one primary antibody (e.g. murine IgG_1) as described, and wash.
3. Stain with a titrated amount of fluorochrome-labelled anti-isotype reagent, (e.g. anti-mouse IgG_1), and wash once.
4. Block free binding sites with unlabelled IgG_1 (100 µg ml^{-1}, 10 min, on ice). Control the blocking efficiency by staining an aliquot of the cells with a fluorochrome-labelled isotype control antibody, e.g. of the murine IgG_1 subclass.
5. Stain with the second and third haptenized or directly conjugated antibodies as described above.

Absorption of polyclonal reagents

To minimize unwanted reactivities of polyclonal reagents, they can be absorbed prior to use on unstained cross-reacting cells.

1. Use stock solution of staining reagent at about 1 mg ml^{-1}.
2. Add 1–2 volumes of the stock solution to 1 volume of cell pellet. Mix gently and incubate for 1–2 h on ice; mix from time to time.
3. Spin down the cells at 350*g* and remove supernatant.
4. Spin down supernatant at 12 000*g* for 20 min to remove protein

(*contd.*)

aggregates and debris. Titrate stock solution for staining and store aliquots at –70°C.

Exclusion of dead cells

If F2 and F3 are not used simultaneously for surface antigens, or the antigens are expressed mutually exclusively on two subpopulations, PI from an aqueous stock solution (0.1 mg ml^{-1}), at a final concentration of 1 µg ml^{-1} can be added immediately (1 min) prior to analysis in order to exclude dead cells. (*Note:* Not for fixed cells.)

Exclusion of non-nucleated cells

LDS 751, a vital DNA dye emitting in the far red, can be used to label all nucleated cells (stock solution 1 mg ml^{-1} in methanol; dilute 1 : 100). Add the dye immediately before analysis (Recktenwald, 1988). It also can be used to identify cells, in fixed samples, which were dead prior to fixation (Terstappen *et al.*, 1988).

Intracellular Staining

Basic principles

The basic principles of intracellular staining are discussed in the chapter by Yssel and Cottrez in Section III. A protocol for staining of murine cytokines and a list of appropriate reagents is given here. Specificity of staining can be controlled by staining of cytokine transfectants and blocking with soluble cytokine (see earlier). In addition, the intracellular staining of most cytokines is concentrated in the Golgi compartment. Upon microscopic analysis, this spot-like staining provides further evidence for specific staining. Intracellular staining of cytokines can also be controlled functionally, by using 'cellular affinity matrix technology', which allows isolation of live cells secreting particular cytokines (see later). Such cells are specifically stained for the same cytokine intracellularly, and only these cells secrete this cytokine, as has been shown by sorting, subsequent culture and ELISA. This demonstrates the comparable sensitivities of these techniques.

Standard protocol for intracellular staining of murine cytokines

Antibodies for intracellular staining of murine cytokines

See Table 2.

Fixation

1. Wash cells once with PBS and resuspend in PBS at $2 \times 10^6 ml^{-1}$.
2. Add 1 volume of 4% formaldehyde/PBS. Incubate for 20 min at room temperature.
3. Wash twice with PBA.

(*contd.*)

4. Resuspend in PBA at 1×10^6 to 2×10^6 cells ml^{-1} and store at 4°C in the dark until staining.

Intracellular staining of one cytokine and one surface marker

1. Use about 1×10^6 fixed cells for each 1.5-ml test-tube sample. Spin down for 10 min at 300*g*.
2. Incubate pellet with 50 µl digoxigenin (DIG) conjugated anti-cytokine antibody and PE-labelled antibody against a surface antigen in PBA/0.5% saponin (from Quillaja bark; S-2149, Sigma, St Louis, MI, USA) ('saponin buffer') for 10–15 min at room temperature.
3. Wash twice with 1 ml saponin buffer.
4. Incubate pellet with 50 µl sheep anti-DIG-FL (Boehringer Mannheim) in saponin buffer for 10–15 min at room temperature.
5. Wash with 1 ml saponin buffer and resuspend pellet in PBA.
6. Flow cytometry or fluorescence microscopy.

Table 2. Antibodies for intracellular staining of murine cytokines

Cytokine	Antibodies	Cytokine	Antibodies
IFN-γ	R4-6A2 or AN18.17.24	IL-4	11B11 or BVD4-1D11
IL-2	S4B6	IL-5	TRFK5
IL-3	MP2-43D11 + MP2-8F8	IL-10	JES5-2A5

For a description of cytokine antibodies, see the Pharmingen catalogue.
For a description of AN18.17.24, see Prat *et al.* (1984).

Flow Cytometry of Secretion

Basic principles

The affinity matrix (see earlier) is created by biotinylation of cell surface proteins and labelling with an antibody–avidin conjugate ('catching antibody', see Manz *et al.* (1995); for the preparation of protein–protein conjugates in general, see Hermanson (1996)). Pairs of antibodies that recognize two different epitopes of the secreted molecule are used for the secretion assay. During labelling of the cells, secretion is prevented by sodium azide (0.02%) and incubation on ice. The cells are then allowed to secrete their product for a defined time at 37°C. The required time for secretion depends on the rate of secretion, and may vary from 15 to 60 min. To prevent diffusion of secreted proteins from secreting to non-secreting cells, for secretion the cells are incubated in a medium of high viscosity (30–40% gelatine or 4% alginate). Alginate forms a gelatinous precipitate with Ca^{2+}, which can be dissolved by addition of EDTA. Gelatine can be dissolved by adding warm medium to recover the cells after secretion. After secretion, the cells are collected and stained with the second affinity label for the secreted product, which is bound to the affinity matrix on the surface of the cells.

Protocol: cellular affinity matrix
(By R. Manz and M. Assenmacher.)

Antibodies

See Table 3.

Preparation of low-permeability medium (gelatine)

1. Dissolve 60 g gelatine (type B, from bovine skin 75 Bloom) in PBS to a final volume of 100 ml, at 40°C.
2. Dialyse extensively against PBS and once against RPMI 1640 for several hours at 4°C. Check for toxicity by incubation with test cells.
3. Store aliquots at –20°C.

Biotinylation of cells

1. Wash cells (10^7–10^8) once with PBS/BSA. Keep an aliquot (control 1).
2. Dissolve 1 mg ml^{-1} LC–biotin–succinimidyl ester in PBS pH 8.5.
3. Immediately resuspend the cells in the biotin solution (10^7–10^8 cells per 0.2–1 ml).
4. Incubate for 10 min at room temperature.

For the following steps, keep the cells on ice to prevent secretion.

5. Wash twice with 50 ml cold PBA, using new tubes for each washing step. Free biotin must be removed completely. Keep two aliquots (controls 2 and 3).

Affinity matrix

1. Blocking of membrane bound molecules: incubate biotinylated cells with unlabelled detection antibody (20–50 µg ml^{-1}). Wash with PBA. Take one aliquot (control 4).
2. Incubate cells with titrated amount of avidin–antibody conjugate for 5–10 min on ice, in the presence of unconjugated catching antibody (100–400 µl). Keep two aliquots (controls 5 and 6).

Incubation for secretion

Gelatine
1. Mix cells with 2–10 ml RPMI/40% gelatine/10% fetal calf serum (FCS), prewarmed to 37°C, and plate on Petri dishes.
2. Incubate at 37°C for 10–90 min.
3. Mix with a 5- to 10-fold volume of PBS (37°C) and transfer to a new tube containing the same volume of cold PBS (10°C).
4. Spin down at 10°C.

Alginate
1. Mix the cells with 1–5 ml RPMI/4% sodium alginate/20% FCS.
2. Inject into 20–30 ml 45 mM $CaCl_2$/100 mM NaCl (26G needle).

(*contd.*)

3. Aspirate supernatant, add 10–20 ml RPMI/10% FCS and incubate for 10–90 min at 37°C.
4. Add 5–20 ml RPMI/80mM EDTA, pH 7.0, at room temperature, until precipitate is dissolved.
5. Wash with cold PBS/BSA.

Detection

Take up cells in 100–400 µl detection antibody (labelled with fluorochrome or hapten) and proceed as described for surface staining.

Controls

- 1 and 2: Check for biotinylation by staining with streptavidin–FL. There should be a 3–4 log difference between the positive (2) and negative (1) samples.
- 3 and 4: Check the density of the affinity matrix with a secondary fluorochrome-labelled antibody against the 'catching antibody'.
- 5 and 6: Check the capacity of the affinity matrix by incubating control 6 with high concentrations of the secreted molecule for 10 min on ice (positive control). Wash and stain controls 5 and 6 with detection antibody.

Controls 1, 3 and 5 serve as negative controls for each staining.

Table 3. Antibody pairs for analysis of secretion of murine cytokines or immunoglobulins

Secreted molecule	Antibody pair (catching antibody, detection antibody)
Immunoglobulin	Rat anti-mouse κ-light-chain (e.g. clone R33-18.10) + rat anti-mouse Ig subclass
IL-2	JES6-1A12 + JES6-5H4
IFN-γ	R4-6A2 + AN18.17.24

For a description of cytokine antibodies, see the Pharmingen catalogue.
For a description of AN18.17.24, see Prat *et al.* (1984).

Detecting Rare Surface Molecules by Magnetofluorescent Liposomes

Tips and tricks

Labelling with liposomes in general follows the rules described earlier. For indirect labelling with liposomes, the primary labelling reagent has to be highly specific and staining conditions have to be optimized in order to minimize background. Free primary or secondary reagents have to be removed carefully. Cells are washed twice after primary staining and liposomes are washed once prior to use. After staining, free liposomes have to be removed carefully by repeated washing (2–3 times). Single liposomes can be detected by flow cytometry, resulting in false-positive signals by coincidental measurement of cells with free liposomes. For the same reason, the flow speed of the sample should be reduced to 100–500 events s^{-1}. On the other hand, cells labelled with liposomes should be resuspended gently after washing, since cell-bound liposomes are sensitive to shearing forces.

Staining with liposomes

1. To decrease non-specific binding, cells are preincubated for 10 min with a 100- to 1000-fold excess of unspecific 'blocking' IgG, kept strictly on ice for the entire procedure, and 0.05% NaN_3 is added to the buffer (PBA).
2. Cells are labelled with haptenized antibody (usually 1–10 $\mu g\ ml^{-1}$) for 10 min on ice.
3. Wash *twice* to remove any free antibody.
4. Liposomes conjugated to hapten-specific antibodies are spun down at approximately 12 000*g*, for 10 min, directly before use and resuspended in PBA. The optimal concentration has to be determined by titration beforehand.
5. The cells are resuspended in the liposome solution. Maximum concentration is about 10^7 cells per 200 µl, with 200 µl minimum volume. Round-bottomed tubes are used for staining and placed on a shaker for at least 30 min on ice. Make sure that the mixture is agitated.
6. Cells are then washed carefully at least twice.
7. Analysis by flow cytometry (flow rate 100–500 events s^{-1}) or isolation of labelled cells by MACS.

Surface cytokine detection and sorting

Some cytokines, such as γ-interferon (IFN-γ) (Assenmacher *et al.*, 1996) and interleukin-10 (IL-10) (Scheffold *et al.*, unpublished), are expressed specifically on the surface of cells secreting those cytokines. They can be used to identify and isolate viable cells according to cytokine expression, e.g. T-helper 1 (Th1) cells secreting IFN-γ. Surface cytokines are expressed transiently during secretion, restricting their use to identification of cells activated *in vivo* or *in vitro* (for activation protocols see the chapter by Yssel and Cottrez in Section III). For staining, antibodies are used that do not recognize receptor-bound cytokines, i.e. antibodies that block cytokine function, such as AN18-17.24 and GZ4 for murine and human surface IFN-γ, and JES5-2A5 for murine surface IL-10. Cells are stained indirectly with digoxigenized cytokine-specific antibodies and anti-digoxigenin liposomes, as described above. Specificity of staining can be controlled functionally by sorting and subsequent culture and ELISA or fixation and intracellular staining. Direct correlation of intracellular and surface staining, prior to sorting, is not possible because liposomes are lysed by saponin buffer.

Analysis

Liposomes are 100- to 1000-fold more sensitive than conventional staining reagents, allowing detection of as few as 50–100 molecules per cell. For analysis of cells stained with liposomes, some factors must be considered. The high sensitivity of the technology requires stringent controls, i.e. cells stained with liposomes alone and an isotype-matched digoxigenized

control antibody. The latter provides the basis for statistical evaluation. One bound liposome is sufficient to stain a cell clearly for cytometric evaluation. For rare antigens of less than 300–400 molecules per cell, cells will be labelled with few liposomes. Some false-negative cells may then occur, i.e. although a subpopulation of cells is separated from negative cells, some positive cells remain unstained (no liposome), although they have similar antigen density.

◆◆◆◆◆◆ CELL SORTING BASED ON AFFINITY LABELLING

Introduction

Basic considerations

Affinity labelling, apart from its analytical potential, allows cells to be isolated according to the label for further analysis. Fluorescence-activated cell-sorting (FACS) and high-gradient magnetic cell separation with superparamagnetic colloids (MACS system) are both based on quantitative labelling. Other, more qualitative methods based on physical (density centrifugation), biochemical (adherence L-leucine methyl ester (LME) lysis) or immunological differences (panning, macroscopic magnetic beads) may be useful for pre-enrichment. Some of these methods are described in the chapter by Czuprynski and Brown in Section II and by Esser (1992).

Sorting strategies

MACS or FACS

For FACS, closed systems with piezo-controlled fluidic deflection systems (Partec, FACSort, FACSCalibur), which provide sort rates of below $10^6 h^{-1}$, and free flow in air systems with deflection of cells in droplets (Coulter EPICS, FACS Vantage, FACStar), with sort rates of more than $10^7 h^{-1}$, are available. All systems allow sorting of cells according to light scatter and up to four fluorochromes. The basic principle of free flow in air sorters is the deflection of droplets containing single cells from the bulk flow by electromechanical or electrostatic means according to their fluorescence or light scatter. They can analyse and sort up to 10^7 cells per hour with high fidelity, and even higher rates can be achieved with high-speed sorting devices, but at lower purity. Higher sorting rates cannot be used since the physical stress of acceleration within the sorting nozzle would damage the cells. FACS is the technology of choice for isolation of cells according to complex multiparameter labelling, intracellular staining and staining for biochemical parameters and DNA content. It provides the option to deflect individual cells or defined cell numbers and can separate cells according to subtle differences in labelling. High-gradient magnetic cell sorting sorts cells in parallel rather than sequentially. Therefore, for

MACS the sort time is independent of the total cell number. This makes it a useful tool for isolating rare cells. MACS can also be used to separate cells according to multiparameter labelling, by sequential separation (see later). Sorting for quantitative differences in magnetic labelling requires fine tuning of the labelling and separation conditions (see below). While MACS is an inexpensive, easy to use system that imposes little stress on the cells, free flow in air systems require an experienced operator for alignment and sorting. Acceleration of cells in the nozzle is stressful for the cells. Aerosol formation increases the risk of infection. Nevertheless, these methods offer unique technical possibilities.

Positive or negative selection?

The first decision in developing sorting strategies is whether to sort for the wanted cells or against all other cells. Positive selection is usually more efficient in terms of purity. However, it requires a specific cell-surface marker for the target cells, which upon cross-linking by labelling should not interfere with cell function.

Negative selection of distinct cells, by depletion of other cells, would leave the enriched cells 'untouched', but the population would be less pure, because it is difficult to find labelling markers for all unwanted cells, especially rare cells.

Analysis

Sorting experiments are evaluated by cytometric analysis of the original, negative and positive fractions. The absolute numbers of (live) cells should be determined in all fractions (e.g. by counting in a Neubauer chamber), so that the recovery rates can be calculated.

Sterility

MACS columns are provided sterile and the sorting procedure can easily be performed under sterile conditions. Sterile FACS sorting requires sterilization of the entire flow system.

FACS

The basic principles of free flow in air sorting is described in detail elsewhere (Melamed *et al.*, 1990; Radbruch, 1992). In any case, it is advisable to use this technology only in well-managed flow-sorting laboratories.

MACS

Principles of magnetic separation

The basic idea of magnetic separation in high-gradient magnetic fields is to combine the advantages of labelling of cells with small, superparamagnetic particles with separation on a ferromagnetic matrix, magnetized by insertion

into an external magnetic field. Cells labelled with superparamagnetic beads are attracted to the ferromagnetic matrix by the magnetic gradient generated. Unlabelled cells are eluted by washing. When the column is removed from the magnetic field, labelled cells can be eluted from the column.

Parameters of MACS sorting

Factors that have to be considered for magnetic sorting include the quality of magnetic labelling and the choice of separation columns.

Magnetic label

The strength of magnetic labelling influences the efficiency of the separation. Like fluorescent staining it is proportional to the density of antigen and, similarly, indirect reagents are more effective than direct labelling. Optimum concentrations have to be checked by titration.

Any background staining will lead to non-specific retention. Single-cell suspensions are essential to prevent clogging of the columns. Magnetic labelling should be performed in a refrigerator (6–12°C), since incubation on ice has been shown to interfere with labelling (e.g. for human CD14).

Separation column

Columns with different capacities and different matrices (wires or balls) are available. The size of the column must be calculated to be able to retain the expected number of positive cells. For positive enrichment, the capacity should be calculated stringently to reduce non-specific retention. For depletion, the column should have higher capacity to guarantee effective retention of all cells. Due to their geometry, ball matrices (RS^+, VS^+, XS^+) have a reduced number of non-specific binding sites, a smaller capacity and a weaker magnetic gradient. They are ideally suited to the isolation of populations of high purity, especially if the total number of positive cells is small. Wire matrices (AS, BS, CS) allow more efficient depletion of cells and can also be used for positive enrichment of cells present in large numbers.

Apart from the choice of column, the speed of flow, which is regulated by flow resistors, has a strong influence on sensitivity and purity. High flow rates will not allow retention of weakly labelled cells, and thus give reduced background and high purity of the positive fraction ('enrichment'). However, not all positive cells will be retained. Slower flow rates will result in quantitative retention of labelled cells (depletion). Two successive rounds of MACS, depletion and positive enrichment can be combined to obtain pure positive and negative populations.

Dead cells

Dead cells take up magnetic beads very efficiently and are therefore enriched non-specifically by MACS. They should be removed prior to MACS, especially for the separation of rare cells.

Sensitivity

The sensitivity of magnetic separation is comparable to fluorescent staining, i.e. clearly stained cells can be isolated by MACS. Because the cells have no magnetic moment *per se*, magnetic sorting allows the separation even of populations that overlap in fluorescent staining due to weakly expressed antigens, autofluorescence and natural CV (see above), if the magnetic label has been titrated carefully.

Multiparameter magnetic cell sorting

Magnetic sorting according to more than one parameter can be done by combining depletion for one parameter and subsequent positive enrichment for a second parameter. Stringent depletion conditions are required to avoid enrichment of cells left over from the first labelling in the second round. For the enrichment, conditions should favour high purity, e.g. high flow rate and ball-matrix columns. This approach has been used efficiently in, for example, the isolation of fetal cells from maternal blood (Buesch *et al.*, 1994). The positive enrichment of cells according to several surface markers can be done using MACS Multisort reagents, which allow enzymatic cleavage of the magnetic beads after separation and subsequent positive or negative enrichment steps. One example for double positive enrichment, the purification of naive T cells from murine splenocytes, is given below.

Isolation of naive murine T cells

Detailed protocols for MACS are supplied together with the reagents. Here, we give a protocol for sorting of naive murine T helper cells according to the expression of CD4 and CD62L (L-selectin (Gallatin *et al.*, 1983)) (protocol by M. Assenmacher and M. Loehning).

Reagents

Antibody conjugates:

- anti-murine CD4 mab: GK1.5 FITC (isomer 1, e.g. Sigma F-7250)
- anti-murine CD62L mab: MEL-14 biotin
- streptavidin phycoerythrin (PE).

Magnetic cell separation reagents (Miltenyi Biotec, Bergisch Gladbach, Germany):

- anti-FITC (isomer-1) MultiSort Kit
- streptavidin MicroBeads.

MACS columns for positive selection:

- MS^+/RS^+ column with MiniMACS (for up to 10^7 positive cells)
- VS^+ column with MidiMACS, VarioMACS or SuperMACS (for up to 10^8 positive cells).

Protocol

First enrichment: separation of $CD4^+$ cells

1. Prepare single-cell suspension of murine spleen cells, wash and stain with anti-CD4 FITC in PBS/BSA as described.
2. Wash once.
3. Incubate pellet with anti-FITC MultiSort MicroBeads (1 : 5 at about 2×10^8 to 5×10^8 cells ml^{-1} in PBS/BSA) for 15 min at 10°C.
4. Wash cells in PBS/BSA.
5. Resuspend cells in PBS/BSA (MS^+/RS^+ columns, 0.5–1 ml; VS^+ columns, 1–2 ml) and apply cell suspension onto the appropriate MACS column. Keep an aliquot (original fraction).
6. Rinse with PBS/BSA (MS^+/RS^+, 3×0.5–1 ml; VS^+, 5×3 ml). Keep an aliquot (negative fraction).
7. Remove columns from magnetic separator and elute $CD4^+$ cells with PBS/BSA (MS^+/RS^+, 1–2 ml; VS^+, 5 ml). Keep an aliquot (positive fraction 1).
8. Check purity of positive cells by flow cytometry. Separation may be repeated to increase purity to more than 98%.

Detachment of magnetic beads

1. Incubate the $CD4^+$ cell fraction with MACS MultiSort Release reagent and 20 µl ml^{-1} cell suspension for 10 min at 10°C.
2. Apply cell suspension onto a MS^+/RS^+ column, in order to remove any remaining magnetically labelled cells. Rinse the column and wash the released fraction.
3. Incubate released $CD4^+$ cells with MACS MultiSort Stop reagent, 30 µl per 1 ml cell suspension, at 2×10^8 cells ml^{-1}, PBS/BSA during the following labelling step.

Second enrichment: separation of $CD4^+CD62L^+$ cells

1. Stain $CD4^+$ fraction with Mel-14 biotin, as described.
2. Wash cells in PBS/BSA.
3. Incubate pellet with Streptavidin MicroBeads (1 : 10 at about 2×10^8 cells ml^{-1} in PBS/BSA) for 10 min at 10°C.
4. Add streptavidin PE; 5 min, 10°C.
5. Wash cells in PBS/BSA.
6. Resuspend cells in PBS/BSA, apply cell suspension onto the MACS column and proceed as described above.
7. Analyse by flow cytometry.

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2 Measurement of Cellular Proliferation

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INTRODUCTION

Activation and proliferation, along with differentiation into effector cells, are important responses of the cells of the immune system to a challenge by invading pathogens. Cellular expansion is meant to increase the number of effector cells capable of generating both the necessary inflammatory milieu and an increased frequency of pathogen-specific responder cells.

Depending on the nature of the pathogen and the challenge to the organism, the response may differ considerably in terms of the cellular components involved and the appropriate effector functions chosen by the immune system. For instance, cytotoxic T lymphocytes (CTLs) usually appear during viral infections. T-helper 1 (Th1) cell responses are induced by intracellular bacteria such as mycobacteria, while Th2 patterns can be typically detected in connection with helminth infections, and humoral (B cell) immune responses are frequently observed following immunizations with protein. Moreover, co-operative action is a characteristic but still not fully understood aspect of the immune system, involving the various components during maturation of the immune response to varying degrees. Thus, proliferation is a frequent response of T lymphocytes to antigenic stimulation.

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In vitro experiments are an important supplement to studying infectious diseases. Proliferation studies are performed *in vitro* using isolated and highly purified lymphocyte subsets in order to dissect cellular complexities and to focus on the particular roles of the individual subsets. Therefore, studies with isolated cell subsets involve a risk of missing or underestimating potentially critical regulatory cell interactions.

The principle of all *in vitro* measurements of cellular proliferation is to detect cell division and cell growth as a result of activation and certain culture conditions. Frequently these culture conditions are stimulatory, especially when using primary ('resting') lymphocytes. Cell-based bioassays are often used to detect soluble mediators by means of a characteristic growth factor dependency of indicator cell lines. Here, cellular proliferation is used as a read-out to identify and quantify soluble mediators. Finally, the inhibition of proliferation (by suppression, anergy or apoptosis) has aroused increasing interest in recent years. In these cases, the decrease in the growth rate and/or cellularity reflects the efficiency of growth inhibition. The latter two applications are normally carried out using preactivated cells. As a general rule, comparability and interpretation of proliferation data requires that the cellular growth response or the culture conditions be standardized.

The aim of this chapter is to provide an overview of various techniques used to assess the growth pattern of lymphocytes. We will focus primarily on [^{3}H]thymidine incorporation into newly synthesized DNA of dividing cells, cleavage of the membrane permeable tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to measure metabolic activity, and the enumeration of absolute numbers (cellular expansion) of subpopulations using a modified flow cytometry method, termed standard cell dilution assay (SCDA). As the basis of these methods varies, each one can be considered representative of a few other alternative approaches, some of which are mentioned but cannot be discussed in greater detail.

◆◆◆◆◆◆ [^{3}H]THYMIDINE INCORPORATION

Principle

Following appropriate stimulation, lymphocytes start to expand vigorously by repetitive mitosis cycles. Each cycle of cell division requires cell cycle progression and DNA synthesis. Basically, each cell of a given population synthesizes a similar amount of DNA prior to its division. Thus, expansion of a cell population correlates quantitatively with its rate and extent of DNA synthesis. This basic relationship led to the wide application of assessing DNA synthesis as a measurement of cellular proliferation. The labelled nucleotide, [^{3}H]methylthymidine ([^{3}H]TdR), at a sufficiently high concentration (i.e. 2–3 nM, 0.2 Mbq ml^{-1}) competes with endogeneously available thymidine for incorporation into newly synthesized DNA (Strong *et al.*, 1973). Convenient quantification is achieved by detecting radiolabelled DNA using glass-fibre filter mats, which trap genomic DNA along with some insoluble cell fragments, but spare oligonucleotides or single bases.

Equipment and reagents

- Tritiated methylthymidine ([^{3}H]TdR) as a stock solution of 37 MBq ml^{-1} (1 mCi ml^{-1}) (Amersham, Du Pont NEN).
- Plain RPMI 1640 or comparable culture medium without any source of serum to prepare [^{3}H]TdR working dilutions.
- Multichannel pipettes and/or single-tip repetitive dispensers are extremely useful for handling multiple microtitre plates.
- Cell harvester unit with suitable glass-fibre filter mats are frequently used to conveniently harvest 96-well microtitre cultures in a single aspiration and washing cycle. Other instruments harvest 12–24 wells per cycle.
- β-Emission counter: Basically, two types of counters are available. (1) liquid scintillation counters that detect light generated in response to β^- particle energy absorption by scintillator atoms. The light intensity measured correlates directly with β emission of the total DNA-bound [^{3}H]. (2) β Radiation surface detectors, which do not use scintillation fluid, but rather measure the short-range β radiation emitted by the radionucleotides collected directly on top of the filter mats by stationary devices or movable scanners, which are both equipped with high-sensitivity detectors for β radiation. Instruments for cell harvesting and β counting can be purchased from various companies (e.g. Wallac, Packard).
- Appropriate containers for collecting liquid (scintillation fluid or culture medium) or solid radioactive waste and a disposal facility for radioactive materials.

Assay

Sterile techniques must be used throughout, and caution must be exerted when handling radioactive materials.

1. [^{3}H]TdR working solution at a 1:20 dilution in RPMI (i.e. 0.2 MBq ml^{-1}, 50 μCi ml^{-1}) without any source of serum can be prepared in advance and stored for several weeks.
2. 96-well microtitre plate cultures are [^{3}H]TdR labelled by adding 0.037 MBq per well (1 μCi per well, 20 μl working solution) and incubated for an additional 6–18 h at 37°C before harvesting. The optimal incubation period for individual applications should be established in pilot experiments, although in general there is little change in [^{3}H]TdR uptake within 6 and 18 h of radioactive labelling.
3. Cultured cells can be harvested by aspirating and washing out the contents of the microcultures onto a glass-fibre filter mat using an appropriate cell harvester. Alternatively, it is safe to freeze the whole [^{3}H]TdR labelled microtitre plate at –20°C for transient storage purposes if desired. After thawing, cultures can be harvested and processed as above.
4. Glass-fibre filter mats must be completely dry before counting. They can be air dried overnight at room temperature or in a dry incubator. If immediate counting is required, filter mats can be dried efficiently using a microwave oven. Depending on the instrument used, filter mats are either soaked with scintillation fluid or directly counted according to the manufacturer's recommendations.
5. Radioactivity is measured as counts per minute (cpm) and averaged to disintegrations per minute (dpm). The dpm of experimental cultures are usually compared to [^{3}H]TdR labelled control cultures devoid of any responder cells or containing unstimulated cells.

Troubleshooting

False-negative measurements can occur due to failure to collect all the cells from the culture well, or they can result from the generation of apoptotic DNA fragments of newly synthesized [^{3}H] labelled DNA, which are not efficiently retained by the filter mats. Obviously, cell-collection failure can reflect a mechanical problem, e.g. one or several aspiration nozzles or tubes may be blocked, preventing effective aspiration and washing of the culture wells. In this case, individual parts or the whole harvester circulation can be flushed with an appropriate cleaning reagent. Alternatively, culture-well rinsing may be inadequate, especially if adherent cells are used. If microscopic examination of individual culture wells reveals residual plastic adherent cells after the first harvest, an additional trypsin/ethylenediaminetetraacetic acid (EDTA) detaching step is recommended, followed by a second harvest cycle onto another filter mat. In this case, the dpm of the corresponding culture-well positions should be added up.

It is possible that the glass-fibre filter mats do not collect all DNA-bound radioactivity. This can be due to: (1) insufficient thickness of the filter mats, which can be explored by using two filter mats and counting the upper one which should not show any radioactivity; or (2) stimulated cells may undergo apoptosis, after a characteristic period of growth and DNA synthesis, resulting in oligonucleosomal fragmentation of their DNA. Fragments may be too short to be retained efficiently by the filter mats. In fact, an apoptosis assay has been designed to exploit exactly this feature. It measures the reduction in [^{3}H] TdR activity collected by the filter mats from prelabelled cells exposed to an apoptotic stimulus (Matzinger, 1991). The presence of apoptotic cells can be confirmed using several flow cytometric approaches for the detection of apoptosis (see below and other publications).

Application

Although [^{3}H]TdR incorporation assays can be used almost universally to detect eucaryotic cell proliferation *in vitro*, there are some limitations to this. Firstly, and most importantly, [^{3}H]TdR uptake does not allow the measurement of individual rates of DNA synthesis, which can occur among various subsets in heterogeneous cell populations. The [^{3}H]TdR incorporation estimates the average overall DNA synthesis of the cell population. An alternative approach uses bromodeoxyuridine (BrdU), a thymidine analogue which is similarly incorporated into DNA (Crissman and Steinkamp, 1987), and can be detected by flow cytometry. Although the BrdU assay is more time-consuming, it can be used as an alternative to [^{3}H]TdR uptake under certain conditions, and it is certainly superior to [^{3}H]TdR for *in vivo* labelling studies (Tough and Sprent, 1994).

Secondly, it has been demonstrated convincingly in recent years that proliferation as measured by [^{3}H]TdR does not always reflect cellular expansion quantitatively. For instance, activation-induced cell death (AICD) has been shown to be an important negative regulatory mechanism limiting cellular expansion, especially of T cells (Kabelitz *et al.*, 1995).

Activation followed by cell-cycle progression and DNA synthesis can sensitize T cells to undergo AICD. This in turn can result in an overall increase in [^{3}H]TdR uptake, while at the same time viable cell numbers decrease due to AICD.

◆◆◆◆◆◆ COLORIMETRIC ASSAY: ENZYMATIC CLEAVAGE OF MTT

Principle

MTT is a water-soluble tetrazolium salt, which is widely used to measure the redox potential of cells. The measurement of metabolic activity on the basis of MTT cleavage can be used to assess viability, proliferation or cytotoxicity (Mosmann, 1983). Like other tetrazolium salts, upon reduction MTT forms a brightly coloured formazan compound, which in the case of MTT crystallizes allowing detection either as the precipitate, which can be used to study histochemical localization at the site of reduction, or through photometrical quantification upon solubilization of the crystals in organic solvents. Cells are incubated with the membrane permeable MTT to allow access to microsomal enzymes (dehydrogenases) of the respiratory chain, which cleave MTT to form the purple, insoluble reaction product. Following solubilization, quantification of the coloured cleavage product yields estimates of viability and metabolic activity, which correlate with cell growth.

Equipment and reagents

- MTT (sigma) is solubilized (5 mg ml^{-1}) in phosphate buffered saline (PBS) or RPMI (5 mg ml^{-1}), without phenol red), and filter sterilized (0.2 µm). To avoid spontaneous precipitate formation, aliquots should be frozen if stored for an extended period.
- Acidic isopropanol (0.04–0.1 N HCl in isopropanol) or 0.01 N HCl in Tris/10% sodium dodecyl sulphate (SDS) detergent to solubilize the formazan precipitate.
- Conventional spectral photometric microplate reader (enzyme-linked immunosorbent assay (ELISA) reader) equipped with appropriate wavelength filters of 570 nm (specific reading) and 630–690 nm (background wavelength).

Assay

1. The MTT assay is performed in a flat-bottomed 96-well microtitre plate. Depending on the cell type and assay conditions, 5×10^4 to 5×10^5 cells are cultured in 100–200 µl culture medium per well for the desired periods.
2. Remove and discard culture supernatant so that no more than 100 µl culture medium remains before adding 20 µl MTT solution (final MTT concentration range 0.5–1 mg ml^{-1}).

3. After incubation at 37°C for 4 h a purple-blue formazan precipitate should be visible in wells containing viable, proliferating cells.

The reaction is stopped by adding 100 µl acidic isopropanol or HCl/SDS to each well.

4. The dye crystals solubilize within 30 min after addition of acidic isopropanol. Dissolution will proceed more slowly when HCl/SDS is used. In this case, the plates are incubated overnight at 37°C. After complete dissolution, visual discrimination between positive (purple) and negative (yellow) wells is quick and simple (screening).
5. For dye quantification, microplates can be read on an ELISA reader using a 570 nm filter; 630–690 nm filters should be used for the reference wavelength. The negative control wells (reference wells, blank) contain MTT in culture medium but no viable cells, which should remain yellow. Optical density (OD) readings of strongly proliferating cells can be as high as 0.5–2.0.

It is strongly recommended that the desired responder cells or indicator cell lines be tested for their capacity to reduce MTT in a 4-h assay. This will determine their usefulness for application in an MTT assay. While established cell lines and lymphoblasts generally perform well, we find the MTT assay of limited use for the analysis of primary lymphocytes due to their low metabolic activity.

Applications

MTT has been used extensively in bulk cell viability assays. Its principal application is a fast screening procedure for rapidly growing cell lines, such as immortalized cells or factor-dependent indicator cell lines, which allows a high throughput of samples (Heeg *et al.*, 1985). For these applications, the results of the MTT assay are comparable to [^{3}H]TdR incorporation. This is illustrated in Fig. 1, where the time-course kinetics of proliferation of U937 cells measured by [^{3}H]TdR uptake or MTT assay are compared.

The advantages of the MTT method over other proliferation methods are: (a) no handling of radioactive materials, (b) adherent cell lines can be used as easily as suspension cultures, and (c) fast and easy applicability and high-throughput analysis using a spectral photometer (ELISA reader). Conversely, the nature of the MTT assay (and related assays) readily explains some of its disadvantages: (a) heterogeneous populations are difficult to analyse due to a considerable variability in the contribution of individual cell types in reducing MTT (variable cell size and metabolic activity); and (b) antigen-presenting cells or accessory cells, even if irradiated and thus unable to synthesize DNA, may be radioresistant enough to metabolize MTT to some extent. This can obscure the specific analysis of the desired responder cells. Therefore, under certain circumstances, the applicability of the MTT assay may be limited due to the cell populations under investigation. Although MTT remains the most commonly used tetrazolium salt for application in viability assays, there is another

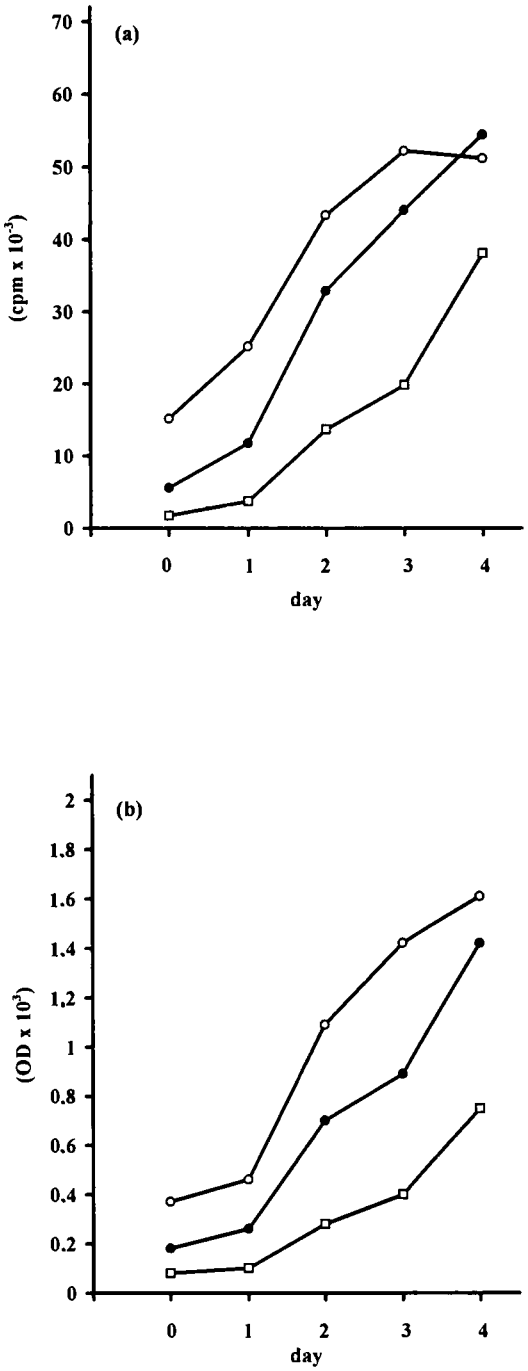

Figure 1. Comparison between [^{3}H]TdR incorporation and MTT assay. Transformed U937 cells were seeded in flat-bottomed microtitre plates in three different concentrations:(□) 5000, (●) 10000 and (○) 25000 per well). Proliferation was monitored over 4 days by labelling culture wells for 4 h with [^{3}H]TdR (a) or MTT (b).

tetrazolium salt, 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5 caroxanilide (XTT), which is soluble in water, and thus does not require a solubilization step prior to analysis. In addition, it has comparable or better sensitivity than MTT in assays that probe the metabolic activity of enzymes of the respiratory chain. Therefore XTT, although more expensive, appears to be a substitute for MTT with some advantages for certain quantitative high-throughput applications. Kits based on MTT, XTT or WST-1 (another modified tetrazolium salt) are commercially available (e.g. Boehringer Mannheim).

◆◆◆◆◆◆ DETERMINATION OF ABSOLUTE CELL NUMBERS: STANDARD CELL DILUTION ASSAY

Principle

Conventional flow cytometry determines the relative frequency of cells using criteria defined by one or more parameters such as cell size (forward light scatter (FSC)), 90° angle light scatter (side scatter (SSC)), and fluorescence label (see the chapter by Scheffold and Radbruch in Section I). The standard cell dilution assay (SCDA) is a modified flow cytometric approach that enumerates phenotypically defined subsets of viable cells (Pechhold *et al.*, 1994a). Basically, SCDA measures the frequency of the cell subset of interest relative to a constant number of reference cells (standard cells) added per sample. The principle is illustrated in Fig. 2. Viable responder cells (to be enumerated) are mixed with a known number of standard cells. From the relative ratio of viable responder cells to dead standard cells, the absolute number of the viable cells of interest (responder cells) can be cal-

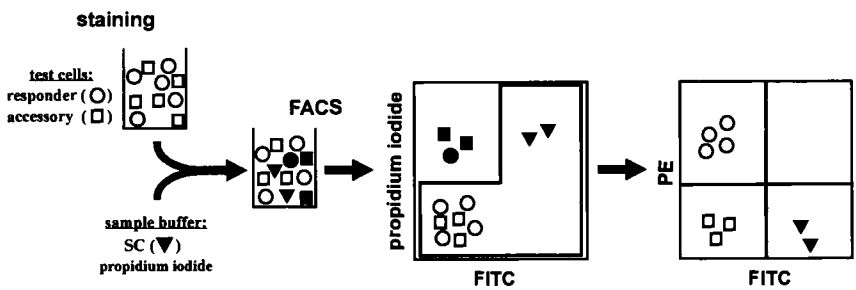

Figure 2. Principle of SCDA. The cell population of interest (responder) is stained by fluoroisothiocyanate (FITC) or phycoerythrin (PE)-conjugated monoclonal antibody (○) in the first step. In a heterogeneous population, bystander (accessory) cells are present (□). Following washing, sample buffer containing standard cells (SC) (▼) and propidium iodide is added in the second step, which labels dead test cells (●,■). Finally, the sample is subjected to three-colour fluorescence-activated cell sorting (FACS) analysis. Two FACS displays of the SCDA sample and the electronic gate used to exclude dead cells are illustrated (FL-3, propidium iodide versus FL-1, SC; and FL-2, PE-stained test cells versus FL-1, SC).

culated. This ratio is not influenced by the absence or presence of additional third-party cells (e.g. accessory cells). A similar principle, for example, underlies some methods of antigen quantification in radioimmunoassays and certain approaches for quantitative polymerase chain reaction (PCR). In the former assay, competitive binding takes place between monoclonal antibodies and a mixture of radioactive labelled antigen of a known concentration and the test antigen. In the latter approach, two slightly different polymerase chain reaction (PCR) templates (i.e. the test template and the control template of a known abundance) compete for binding by the same primer pair, yielding two PCR products of distinguishable sizes. Similarly to SCDA, the ratio of the results obtained from each pair of competitors directly correlates with their relative abundance.

Equipment and Reagents

SCDA requires the basic equipment necessary for staining cells and flow cytometry (see also the chapter by Scheffold and Radbruch in Section I). Briefly, FACS staining is performed using appropriately diluted, fluorochrome-conjugated mAbs and staining buffer (1% bovine serum albumin (BSA) in PBS without Ca^{2+}). Samples can be run on conventional cell sorters and cell scanners such as FACScan, FACSCalibur (Becton Dickinson) and Epics XL (Coulter). Fixation and storage after staining is not possible, as it interferes with propidium iodide (PI) facilitated live/dead cell discrimination.

Standard cell preparation

The main criteria for useful standard cell preparation are:

- Homogeneous FSC and SSC appearance within the range of sample cells.
- All standard cells must stain brightly for any single surface marker or a combination of several surface markers. This labelling is essential, since it allows the discrimination of standard cells from both dead and unstained sample cells (see below).

Standard cell stocks can be prepared from freshly isolated human T cells or from various human or mouse cell lines. We use biotinylated anti-MHC class I mAb W6/32 plus fluoresceinisothiocyanate (FITC)–avidin for human standard cells. The FACS presentation of a typical standard cell preparation is shown in Fig. 3. Standard cells (10^7–10^8) can be prepared and stored at $10^7 ml^{-1}$ in PBS/1% paraformaldehyde (PFA) protected from light at 4°C for several months, allowing utilization in many different experiments. About 10^4–10^5 standard cells per sample are used depending on the staining procedure and the sample size (see below). Standard cell stocks may be counted using a conventional haemocytometer, in order to control cell number stability during storage.

Alternatively, and perhaps more conveniently, fluorescent polysterene beads of approximately 8–10 µm diameter can be purchased from

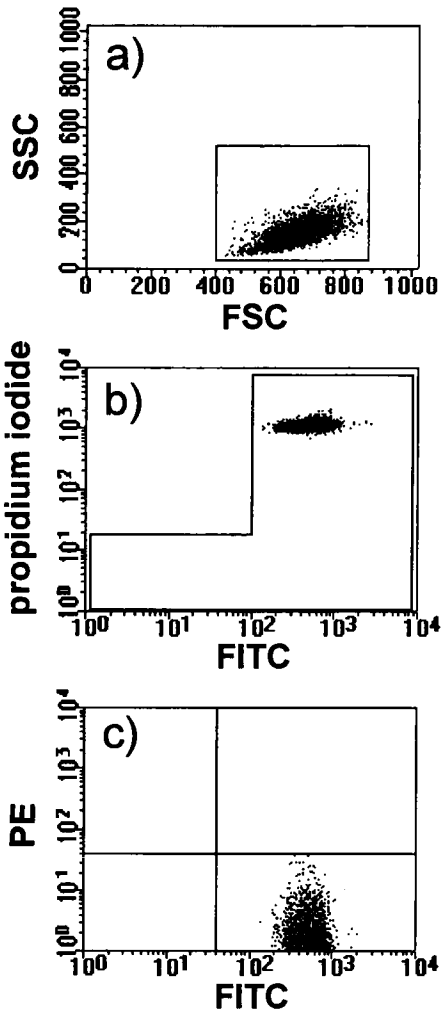

Figure 3. Standard cell preparations for SCDA. Standard cells were stained and fixed as described in the text. The typical standard cell FACS appearance of all five parameters (FSC, SSC and FL-1 to FL-3) available on a conventional FACScan are shown. It is critical that virtually all cells of the standard cell populations have a homogeneous appearance, and stain brightly for FITC.

commercial sources (e.g. Molecular Probes, Polysciences) and be used to replace standard cells. However, modifications in the gate settings may be required, depending on the light and fluorescence characteristics of the individual products. For instance, fluorescent beads tend to display lower FSC (smaller cell size) and much higher SSC characteristics and do not usually fall within the range of sample cells, thus requiring the use of a compound FSC versus SSC gate.

Assay

Sample buffer

The complete sample buffer consists of standard cells at a concentration of 1×10^5 to $5\times10^5 ml^{-1}$ in PBS/1% BSA, and a low dose of PI ($0.2\,\mu g\,ml^{-1}$). The concentration of the standard cells should be chosen depending on the expected number of responder cells being enumerated, which depends on the input cell number and the culture condition used. For statistical reasons, responder cells should not outnumber standard cells by more than 20- to 25-fold. Thus concentrations of standard cells of 1×10^5 to $5\times10^5 ml^{-1}$ are adequate for most assays using cultures of the 96-well format. PI concentrations to discriminate live and dead cells should be as low as possible, because a strong PI fluorescence may interfere in a false-positive manner with phycoerythrin (PE) detection, and renders electronic fluorescence compensation difficult. The sample buffer should be prepared in advance and can be stored at 4°C for a few days. It is important to use the same complete sample buffer if titration experiments or kinetic SCDAs are carried out on different days.

Staining

Staining can be done in duplicates from cells cultured in a 96 microwell plate. Rinsing the culture wells and the tips with PBS is recommended to minimize the loss of cells after their transfer to a staining vessel. It is important to note that only the cells contained in the final, stained sample can be enumerated by SCDA. In most cases, one-colour staining step is performed with the sample cells using a fluorescence label distinguishable from both standard cells and PI-stained dead cells (Pechhold *et al.*, 1994a). For instance, if FITC-stained standard cells are used, PE-conjugated mAbs are appropriate for labelling the sample subpopulation of interest. A direct staining procedure using PE-conjugated primary mAbs is preferable to two-step indirect staining.

We strongly recommend optimizing the staining procedure and handling of multiple samples by using 96 V-bottom microtitre plates for staining. The 96 V plates facilitate the use of multichannel pipettes for transfer of cultured cells, and adding wash or sample buffer. It is also advantageous to use small tubes with a maximum volume of approximately 500 µl placed in 96-well racks, which are available from various commercial sources (e.g. USA-Scientific, Greiner). This system allows multichannel pipette handling, and the tubes can be placed inside a conventional fluorescence-activated cell sorting (FACS) tube (4 ml) for running on the flow cytometer. Note that some sheath fluid backflush aspiration devices are not compatible with the small volume/tube procedure described, and may have to be disconnected temporarily.

FACS measurement

Samples are washed once after staining and resuspended in complete sample buffer. The sample buffer containing the standard cells must be resuspended thoroughly before adding a constant volume (e.g. 100 µl) to each sample. After a short incubation period of 10–20 min, the samples have to be run on the flow cytometer and cannot be fixed and stored. We recommend gentle vortexing of the samples immediately before acquisition.

Data analysis

SCDA data analysis is achieved using a four-parameter gate setting (Pechhold *et al.*, 1994a), as illustrated in Fig. 4. The first gate is applied to FSC and SSC. Similarly to conventional gating by size (FSC) and 90°C

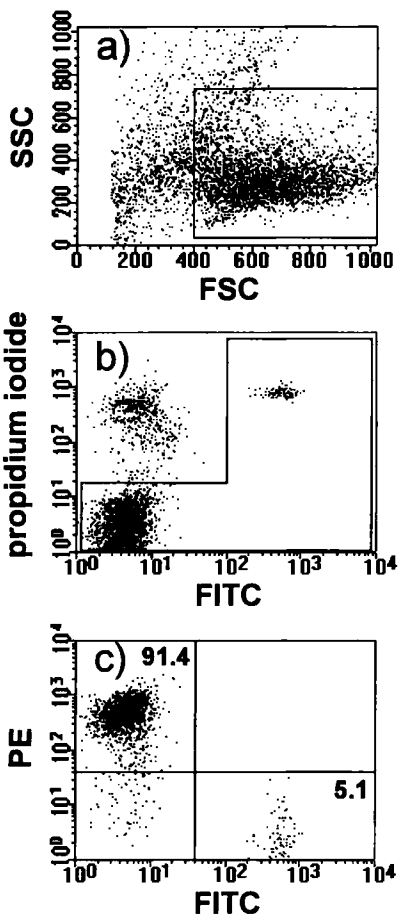

Figure 4. Complete sample cell preparation for SCDA analysis. Human PBL (10^5 per well) were stimulated with phytohaemagglutimin *in vitro*. After 4 days, cells were stained with PE-labelled anti-CD3 monoclonal antibody, mixed with standard cells (100 µl, $10^5 ml^{-1}$ in sample buffer containing 0.2 µg ml^{-1} PI), and run on a FACScan. Data analysis is carried out in three steps as outlined in the text. The electronic gate shown in (a) was activated in (b). The quadrant analysis in (c) is based on both gates (a and b).

angle light scatter (SSC), the SCDA gate is placed more generously to ensure that no viable cells are excluded. However, events with decreased FSC should be excluded by the gate, as they indicate cellular debris (Fig. 4(a), left-hand edge), while cells with moderately increased SSC and FSC should be included, accounting for the spacious right and upper gate edges (Fig. 4 (a)). The superimposed second gate (Fig. 4(b)) is applied to the PI and FITC signal (FL-3 versus FL-1 on a FACScan). The main purpose here is to exclude PI^+ cells (dead cells) but spare PI^+ standard cells, which can be separated from dead sample cells due to their FITC fluorescence. Finally, the relative frequencies of the individual populations, including standard cells and stained and unstained sample cells, are obtained using the quadrant statistic analysis of the PE versus FITC display (F1-2 versus FL-1).

The absolute number of viable cells N_v of the stained subpopulation is calculated using the simple formula:

$$N_v = \frac{f_{sample}}{f_{SC}} \times C_{SC} \times V_{SC}$$

where f_{sample} is the relative frequency of the test sample subpopulation, f_{SC} is the relative frequency of the standard cells, C_{SC} is the concentration of standard cells in the complete sample buffer (e.g. $10^5 ml^{-1}$) and V_{SC} is the sample buffer volume added to each sample (e.g. 100 µl).

The cell number calculation from the example shown in Fig. 4 is carried out as follows: the number of viable cells in the PE-stained test cell population is

$$N_v = \frac{91.4}{5.1} \times 10^5 \times 0.1$$

$$= 1.79 \times 10^5 \text{ per well}$$

The absolute cell numbers of the PE-stained and the unstained samples can both be calculated from the same quadrant analysis data set. Furthermore, a conventional, one-colour FACS analysis (print-out and frequency calculation) can be obtained simply by electronically excluding all PI^+ cells, including the standard cells.

Troubleshooting

Controls

In addition to conventional controls for antibody staining, two types of control are specifically recommended for SCDA:

- Always run plain complete sample buffer (without any sample cells). This should result in an absolute cell count of substantially less than 1% of the standard cells ($f_{sample}/f_{SC} < 0.01$, not detectable for instance in the example shown in Fig. 3).

- When cells are activated for a certain period of time, it is helpful to run a SCDA with the input cell population at the onset of the culture period. This will provide an input cell number useful for calculation of the expansion characteristics.
- Measurements in duplicates are sufficient, since a low standard deviation among triplicate or quadruplicate determinations of $< 5\%$ is commonly obtained.

Precautions

The precision of the analysis procedure can be monitored at several levels:

- Preparation of the standard cell stock solution is a critical factor. The fluorescence labelling of the cells must be bright and homogeneous (see Fig. 3 (b,c)), and the concentration precise (count three times independently using a haemocytometer). Counting should be repeated from time to time during storage periods. Note that PFA-fixed standard cells have lost their membrane dye exclusion capability when using vital stains such as Eosin and Trypan Blue.
- Handling of complete sample buffer must be accurate. Thoroughly resuspend the sample buffer immediately before adding the same precise volume to each sample.
- Make sure that all cells are collected from the microculture wells by rinsing the wells and the pipette tips at least once. Any cell loss during handling will result in an underestimation of the real absolute cell numbers.
- Mix the sample cells and the standard cells in the sample buffer by gentle vortexing immediately before making the flow cytometer measurement.

Applications

The intention here is not to list all possible applications of SCDA. Rather, three major assays are described and briefly compared to some other alternative techniques.

Growth characteristics of lymphocyte subsets

It has been difficult to study the antigen-stimulated responses of lymphocyte subsets within heterogeneous cell populations such as peripheral blood lymphocytes (PBL). For instance, protein antigens derived from various bacteria stimulate proliferation of major histocompatibility complex (MHC) class II restricted $CD4^+$ T cells but not MHC class I restricted $CD8^+$ T cells, while $\gamma\delta$ T cells but not natural killer (NK) cells are activated by non-proteinaceous antigens from mycobacteria. Thus, global measurements such as [^{3}H]TdR incorporation cannot easily quantify these responses (Pechhold *et al.*, 1994a,b). Additional measurements such as FACS analysis are required to dissect the involvement of subsets. Two examples of measuring cellular expansion by SCDA are shown in Figs 5 and 6. It can be seen from Fig. 5 that, when PBL are stimulated by killed mycobacteria, the number of viable $\gamma\delta$ T cells expands exponentially over

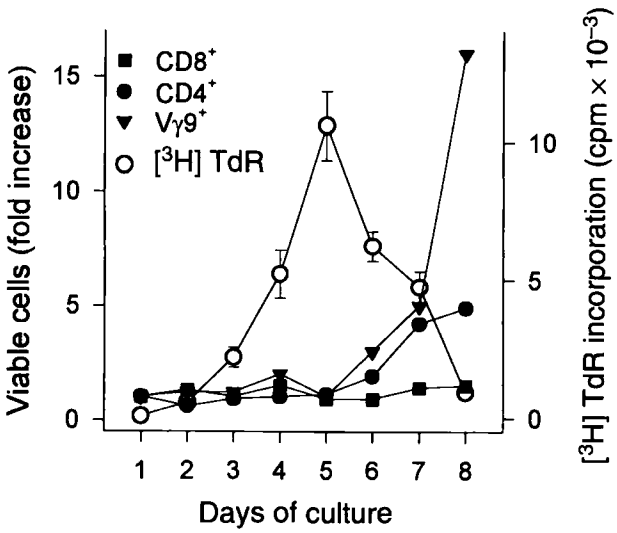

Figure 5. Quantitative analysis of proliferation and cellular expansion of PBL in response to *Mycobacterium tuberculosis*. 10^5 human PBL per microculture well were stimulated with heat-killed *Myc. tuberculosis*. Proliferation, as estimated by [^{3}H]TdR incorporation (○), and the absolute number of viable Vγ9$^+$ γδ T cells (▼), and of CD4$^+$ (●) and CD8$^+$ T cells (■) determined by SCDA were measured daily over a period of 8 days.

8 days, while CD4$^+$ T cell numbers increase only moderately. In contrast, CD8$^+$ T cells do not expand in these cultures. However, the kinetics of [^{3}H]TdR incorporation typically peaks at day 5, considerably earlier than the maximum cellular expansion.

Staphylococcal enterotoxins and related superantigens are known to stimulate only T-cell subsets expressing certain T-cell receptor Vβ elements in a MHC class II dependent fashion, resulting in an apparently linear overall growth of CD4$^+$ and CD8$^+$ T cells. As shown in Fig. 6, SEB and SEE induce preferential expansion of CD4$^+$ T cells among PBL, while the T-cell mitogen PHA triggers stronger expansion of CD8$^+$ T cells.

Activation-induced cell death

T cells can be induced to die upon antigenic or mitogenic stimulation. Thus, quantification of the subpopulations of T cells is a prerequisite for examining activation-induced cell death (AICD) in response to antigen stimulation (Kabelitz *et al.*, 1994) Two general principles have been used to study dying T cells. (1) Detection of programmed cell death or apoptosis, characterized by oligonucleosomal DNA fragmentation into multiples of 180 bp fragments. This fragmentation can be visualized by the TUNEL assay (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labelling) followed by FACS analysis (Gavrieli *et al.*, 1992; Gorczyca *et*

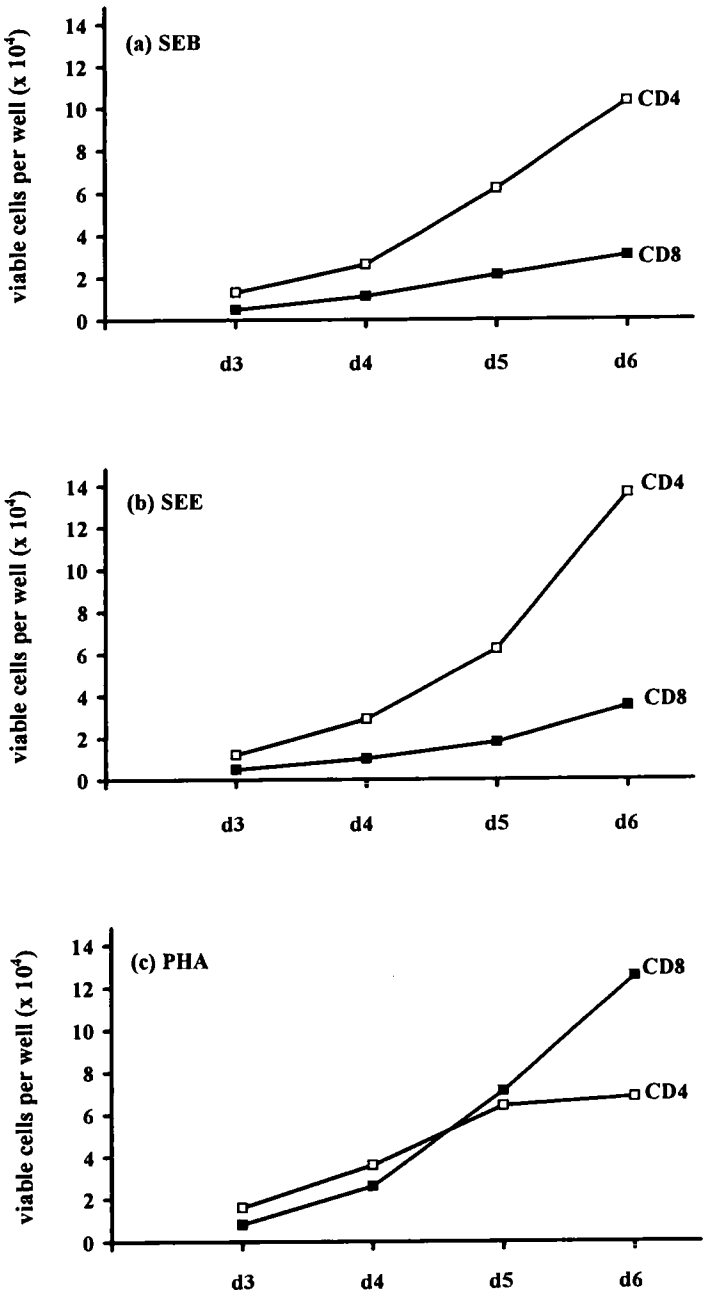

Figure 6. Cellular expansion of $CD4^+$ and $CD8^+$ T cells in response to superantigens SEB (a), SEE (b) or mitogen PHA (c). See legend to Fig. 5.

al., 1993). Other procedures can also detect DNA fragments by flow cytometry using various DNA intercalating dyes, which stain apoptotic cells less intensely (hypodiploid DNA staining) (Nicoletti *et al.*, 1991). (2) Cell death measurement by either PI staining of dead cells (results are difficult to interpret because cells can undergo apoptosis and degrade within hours, thereby losing PI–DNA intercalation), or quantification of viable cells, similar to SCDA. The latter approach allows sample cell acquisition for a constant period of time (i.e. a constant volume) (Boehme and Lenardo, 1993), requires more sample cells in order to be accurate, and a much larger volume per sample. Furthermore, it allows less throughput, and does not therefore offer any advantage over SCDA, except that it does not require standard cells.

Cytotoxicity assays

Studies of cellular cytotoxicity have been commonly performed using ^{51}Cr release assays (Simpson and Chandler, 1986). Despite the requirement of handling the γ-emitting radioisotope ^{51}Cr, the ^{51}Cr release assay is widely used, because it is relatively simple and offers the possibility of handling multiple samples in a minimum period of time. It requires prior labelling of the target cells before interaction with the cytolytic T lymphocytes (CTLs), and release of label after target cell death can be measured in supernatant aliquots.

Disadvantages of the method include: labelling manipulation of the target cells (toxicity) before interaction with the CTL; time limitations, as spontaneous release in the absence of target cell lysis increases considerably with an assay duration >6 h; and the difficulty in labelling certain target cells (slow or non-proliferating cells or adherent cell lines).

SCDA may be useful in certain circumstances, when target cells are used that cannot be loaded satisfactorily with label or when concurrent analysis of both target cell death (cytolytic activity) and responder cell death (apoptosis) are intended. As SCDA does not require prior target cell manipulation, it is suitable for testing cytotoxicity towards adherent target cells without prior detachment, although modifications are required, for two reasons. (1) As many adhering cells usually remain attached to the microculture well after transfer of the cultures into 96V-microtitre plates, an additional 5–10 min incubation step at 37°C using EDTA alone or in combination with low trypsin concentrations is required to collect all adherent cells. Subsequently, cells can be transferred to the staining microplates, mixed with the effector cells of the original microculture, washed, and stained as described above. (2) As many adherent target cells are considerably larger than the CTLs, the gating procedure and analysis may be compounded by different FSC and SSC scatter and autofluorescence signals of both populations.

◆◆◆◆◆ CONCLUSION

The three methods discussed here are based on different principles. Each one has its particular advantages and disadvantages. Thus, the choice of a particular method to measure cellular proliferation and/or expansion depends on the experimental system. A short summary of the most important features of these methods is given in Table 1.

Table 1. Comparison of different methods for measuring cell proliferation and expansion

Method	Advantages	Disadvantages
[^{3}H]TdR incorporation	Sensitive Low background Automated multiwell harvesting and processing	Handling of radioactive substances No information on contribution of subsets in heterogeneous cell populations
MTT assay	No radioactive substances Works well with cell lines No transfer of cells Automated multiwell processing (ELISA reader)	Less suited to analysis of primary lymphocytes (high background) No information on contribution of subsets in heterogeneous cell populations
SCDA	Measures actual cellular expansion Detects contribution of subsets in heterogeneous cell populations No radioactivity	More laborious than [^{3}H]TdR or MTT assays No automated multi-well sampling and processing

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3 Prediction and Determination of MHC Ligands and T-cell Epitopes

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INTRODUCTION

The induction of T-cell response is necessary to most antigen-specific immune responses, especially when the target antigen is a protein. The cell-mediated immune (CMI) response to protein antigens is dependent upon the recognition of the antigen in the form of intracellularly processed peptides, bound to major histocompatibility complex (MHC) class I and class II molecules and expressed at the cell surface. These peptides are defined as T-cell epitopes if the MHC–peptide complex is able to stimulate a cellular immune response through interaction with the T-cell receptor (TCR) of a circulating T cell. Peptides presented in conjunction with MHC class I molecules are derived from foreign or self-protein antigens that have been synthesized in the cytoplasm, and generally range from 8 to 10 amino acids in length (Falk *et al.*, 1991; Matsumura *et al.*, 1992; Germain and Margolies, 1993). Peptides presented in the context of MHC class II molecules are usually derived from exogenous protein antigens, and show a much wider range of reported lengths, from 10 to over 20 amino acids (Rudensky *et al.*, 1991; Chicz *et al.*, 1993b; Srinivasan *et al.*, 1993; Rötzschke and Falk, 1994).

Class I peptides are derived from proteins synthesized in the cytoplasm, including endogenous (self) proteins. Class I peptides are produced by

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proteolytic cleavage in the proteasome. Patterns of cleavage and processing by proteasomes have yet to be fully identified, although there appears to be a strong preference for carboxy terminal hydrophobic or basic residues (Rammensee *et al.*, 1993; Momberg *et al.*, 1994) and γ-interferon (IFN-γ) may influence the patterns of proteasomal cleavage (Gaczynska *et al.*, 1993).

Cleaved peptides are transported into the endoplasmic reticulum by transporter associated with antigen processing (TAP) proteins, coded for by the TAP1 and TAP2 genes located in the MHC class II region of the genome. RMA-S and T2 cell lines, which are TAP deficient, fail to present peptides from the cytosol in the context of MHC class I. The relative abundance of any given peptide in the cytosol, prior to transport into the endoplasmic reticulum (ER) by TAP, may influence the abundance of that peptide within the ER. Peptides may also be excluded by TAP and their availability for binding to MHC molecules in the ER greatly reduced (Momberg *et al.*, 1994). For example, if negatively charged residues in the carboxy terminal position interfere with peptide binding to MHC, such peptides may be excluded by TAP (Momberg *et al.*, 1994; van Endert, 1995). Within the ER, transported peptides, β_2-microglobulin, and MHC class I molecules assemble in a stable complex, and this complex is transported to the cell surface along the secretory pathway. In the absence of peptide, the class I molecule is unstable and rapidly degrades. MHC binding assays, which generally detect the stabilization of MHC class I molecules at the cell surface in the presence of a peptide that binds to the MHC (described in Sette *et al.*, 1987; Kast *et al.*, 1993) have been implemented to screen a set of peptides for those which are most likely to be presented in the context of an MHC molecule to the T-cell receptor.

Class II peptides are generally derived from exogenous proteins, which enter the cell by endocytosis. The synthesis of the class II molecule takes place in the ER. Stabilization of the MHC class II molecules prior to peptide binding is mediated by invariant chain (Ii), which contains a region, CLIP, that blocks the peptide binding groove of the class II molecule. A MHC-encoded accessory molecule, HLA-DM, is required to catalyse the exchange of CLIP for novel antigenic peptides, which compete with CLIP for the binding site. DM appears to favour continued exchange until a higher affinity peptide produces a stable peptide–MHC complex. Some HLA-DR molecules are less dependent on facilitation of peptide binding by DM than are others. Once high-affinity peptides are effectively stabilized within the binding groove, proteolytic degradation of the amino and carboxy terminal residues that are not contained within the binding groove may occur. The peptide–MHC class II complex is then transported to the APC surface along the secretory pathway.

'Cross-talk' between the class I and class II pathways has been observed: peptides derived from proteins synthesized in the cytosol are presented in the context of class II, and peptides derived from exogenous proteins can be presented in the context of class I (Jin *et al.*, 1988; Jaraquemada *et al.*, 1990; Kovacsovics-Bankowski and Rock, 1995).

While antigenic B-cell epitopes are usually composed of amino acid residues, which may be aggregated from various parts of a folded protein (Benjamin *et al.*, 1984), T-cell epitopes are presented to the immune system

as linear peptides of a defined sequence bound to the peptide-binding groove of an MHC molecule. The peptide–MHC complex must interact favourably with the TCR of a neighbouring cell, in order to induce a T-cell response. MHC binding is necessary but not sufficient to generate a T-cell response: recognition of the complex depends on the T-cell repertoire. The final step that all methods for identifying T-cell epitopes have in common is the evaluation of the peptide–MHC complex for immunogenicity *in vitro* (Kast *et al.*, 1994).

In general, fine mapping of T-cell epitopes is accomplished using synthetic peptides and T cells *in vitro*. T-cell proliferation assays, which test for presentation of peptides in the context of MHC class II and recognition of the peptide–MHC complex by T helper cells, are carried out with peripheral blood mononuclear cells (PBMCs), cell lines or T-cell clones. These cells are placed in wells with antigen presenting cells (APCs) and peptide (usually several concentrations are assayed, ranging from 1 μg of peptide per ml to 50 μg ml^{-1}). Stimulation of T helper cells by the peptide–MHC will result in T-cell proliferation and cytokine secretion, measurable by radioactive thymidine incorporation or detection of cytokine release by the activated T cells into the cell culture supernatant (see chapters I.2, II.2.4 and III.5 for more details). Alternatively, peptides can be presented in the context of class I by stimulating with peptide pulsed APCs such as dendritic cells, fresh monocytes derived from peripheral blood, or immortalized B cells obtained by Epstein-Barr virus (EBV) transformation of peripheral blood mononuclear cells, and measuring the lysis of corresponding peptide-pulsed target cells by chromium release assay (see chapters II.2.3 and III.4 for more details).

In summary, the generation of an immune response to antigenic proteins is dependent on four events:

- processing of the protein into peptide fragments in which the epitope is preserved;
- transport within the APC to the intracellular site of binding with MHC molecules;
- binding of the peptide to the MHC molecule, a process which may be facilitated by accessory molecules;
- T-cell recognition of the peptide–MHC complex at the cell surface.

The efficiency with which a potential epitope passes all four hurdles in large part determines its relative immunodominance, compared to other potential epitopes.

◆◆◆◆◆ EXPERIMENTAL AND PREDICTIVE METHODS

Experimental Methods

The overlapping synthetic peptide approach to the identification of T-cell epitopes

Identification of T-cell epitopes within protein antigens has traditionally been carried out through a variety of methods. The most common method, to which all others described in this chapter will be compared, is

the 'overlapping peptide' method. The overlapping method involves the synthesis of overlapping peptides that span the entire sequence of a given protein antigen, and testing of these peptides for their capacity to stimulate T-cell cytotoxic or proliferative responses *in vitro*. One approach might be to synthesize 20 amino acid peptides (20-mers) overlapping by 10 amino acids spanning the entire sequence of a given antigen, particularly if the epitope of interest is thought to be presented in the context of class II molecules. Alternatively, 10 amino acid peptides (10-mers) overlapping by 9 amino acids might be synthesized, if the epitope sought is thought to be presented in the context of a class I molecule. To encompass all possible peptides of length l in the overlapping set, one needs an overlap of l–1 residues. If the sequence of the antigen is of length n amino acids, and the length of the peptides to be made is l, and the length of overlap is m, the smallest number of all possible l-mers spanning the protein one would need to construct and test would be the nearest integer, rounding up from $(n–m)/(l–m)$ (Fig. 1). In the case of class I epitopes, as many as n–9 peptides, overlapping by 9 amino acids, might be synthesized.

Implementation of the overlapping peptide method is cost- and labour-intensive: synthesis of the overlapping peptides can cost between US$10 and US$20 per amino acid. The synthesis of all the overlapping peptides for large protein antigens can be prohibitively expensive. Finally, this method does not guarantee the identification of all possible T-cell epitopes within a protein sequence, as epitopes that bridge overlapping peptides could still be missed, for example, if more than 10 amino acids are required for minimal binding. However, since almost all of the peptides that are potentially T-cell epitopes within the sequence of the protein antigen are assayed, the method can be considered the most *sensitive* method for the detection of T-cell epitopes within a protein sequence.

The recombinant construct approach to the identification of T-cell epitopes

An alternative method is to create recombinant antigens or fragments of antigens and map T-cell epitope to a selected fragment. This approach allows resolution within 20 or so amino acids, depending on the size of the overlapping recombinant fragments. For example, a restriction digest of a given antigen is inserted and expressed in *Escherichia coli*. After purification (and removal of bacterial contaminants such as lipopolysaccharide (LPS)), the expressed protein fragment is evaluated in a T-cell assay. Recombinant fragments that stimulate T-cell responses are mapped; if the recombinatorial fragments overlap, the T-cell epitope can be identified within overlapping segments (Fig. 2). This method requires careful purification of the expressed fragments to eliminate bacterial contaminants, and may not permit precise mapping of the T-cell epitope. However, once the T-cell epitope has been mapped to a selected recombinant fragment, fine mapping using the overlapping peptide method can permit more precise location of the epitope.

Vaccinia constructs expressing fragments of the antigenic protein have also been constructed; such constructs permit the localization of cytotoxic

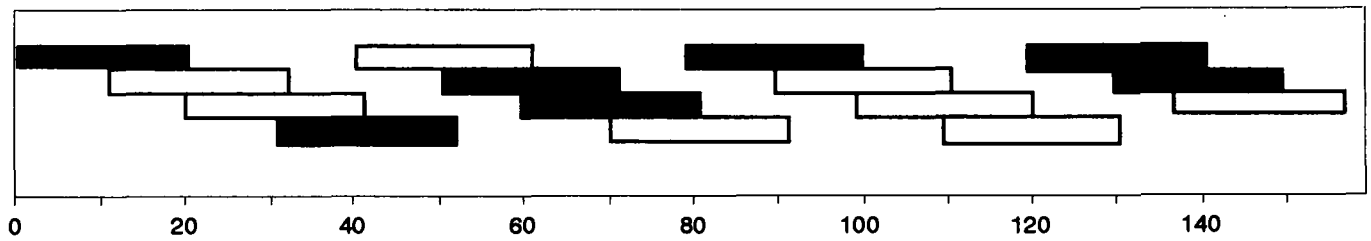

Figure 1. The overlapping peptide method for determining T-cell epitopes as illustrated for the 19-kDa protein from *Mycobacterium tuberculosis*. Peptides of 20 amino acids in length, overlapping by 10 amino acids, would be synthesized, representing the entire sequence of the 160 amino acid protein. A total of 340 amino acids would have been synthesized. Published epitopes are indicated in black.

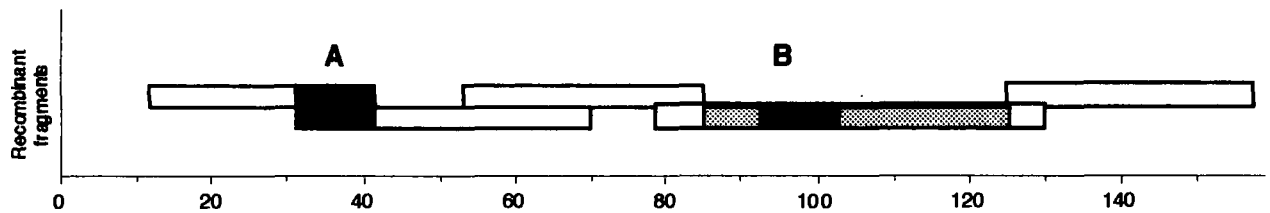

Figure 2. The recombinant fragment method for determining T-cell epitopes. Restriction fragments are expressed and tested *in vitro*. T-cell epitope A would easily be identified, as it is contained within two overlapping recombinant fragments. T-cell epitope B would be more difficult to identify, as only one fragment would have stimulated a T-cell response, and no overlapping recombinant would have stimulated a response; therefore, the T-cell epitope could only be mapped to a region anywhere within the stippled area.

T-lymphocyte (CTL) epitopes within an antigenic protein. Once the subfragment containing the epitope has been localized, the minimal epitope can be defined using overlapping peptides. These approaches reduce the total number of peptides to be synthesized and tested in the search for a T-cell epitope.

Direct deduction of epitope sequences by peptide elution and mass spectrophotometry

Mass spectrophotometry of peptides eluted off the surface of APCs has also been employed to define T-cell epitopes (Hunt *et al.*, 1992). Essentially, large numbers of cells presenting a selected antigen (tumour cells, for example) are cultured, lysed and the MHC molecules purified by binding to allele-specific antibodies. Peptides are acid-eluted from the MHC molecules and subjected to fine fractionation by high performance liquid chromatography (HPLC) and mass spectrophotometry, followed by sequence analysis by mass spectrophotometry. This method has also been used, with minor modification, by Chicz *et al.*, 1993a, b).

Peptides present in femtomolar amounts can be identified in this manner (Hunt *et al.*, 1992). The reconstruction of the peptide sequence is possible in most, if not all, cases. Both peptide epitopes that are naturally derived from antigenic proteins and peptide epitopes that are modified after processing can be identified. This method is exquisitely sensitive, but requires large numbers of cells and very expensive equipment that is not widely available. This approach is most useful for the determination of peptide epitopes derived from tumour cells, particularly when the antigenic sequence is unknown.

Predictive Methods

Pattern-based models for prediction of T-cell epitopes

Pattern based models of T-cell epitope identification are based on the location of simple and localized primary and secondary structural characteristics such as hydrophobicity, helicity or the presence of amino acid 'patterns' or motifs common to peptides that are presented by the MHC. Such local characteristics can often be readily determined from primary sequence, putting the prediction of T-cell epitopes within the reach of simple computer algorithms. In recent years, several computer algorithms have been devised to take advantage of the alphabetical representation of sequence information to search for T-cell epitopes.

DeLisi and Berzofsky (1985) and Rothbard and Taylor (1988) were the first to suggest a conserved pattern of amino acids as a possible predictive tool for antigenicity, based on empirical observations of the periodicity of amino acid residues in T-cell epitopes. Rothbard and Taylor observed a three-residue sequence pattern consisting of a charged residue or glycine, followed by two hydrophobic residues. With modifications based on the actual incidence of these patterns within their database of epitopes, their

final proposed sequence pattern consisted of four or five residues: a charged residue or glycine, followed by two hydrophobic residues, with a hydrophobic residue or proline at the fourth position, and either a polar residue or glycine in the fifth position.

An alternate algorithm, which predicts subsequences of primary structure that have a high probability of folding as amphipathic structures (AMPHI) (Margalit *et al.*, 1987), also achieved a substantial degree of success in the prediction of antigenic sites from primary structure. DeLisi and Berzofsky (1985) originally suggested the hypothesis that T-cell antigenic peptides are amphipathic structures bound in the MHC groove, with a hydrophobic side facing the MHC molecule and a hydrophilic side interacting with the T-cell receptor (Margalit *et al.*, 1987; Spouge *et al.*, 1987). These authors proposed that the potential to form an amphipathic α-helix was characteristic of the majority of known T-cell epitopes.

The AMPHI algorithm used as an input the amino acid sequence of the protein, converted it into a sequence of hydrophobicity values, and searched by power spectrum procedures for segments with the desired periodicity in hydrophobicity. The output was a list of recommended amphipathic peptides, each characterized by a measure of the amphipathicity, the amphipathic index. Another strip-of-the-helix algorithm searching for sequences in which hydrophobic residues would line up along one face of a helix, also correlated with T-cell antigenic sites and detected a similar periodicity of hydrophobicity (Stille *et al.*, 1987; Reyes *et al.*, 1988, 1990). A number of studies subsequently demonstrated significant correlations between antigenic sites and α-amphipathicity (Cornette *et al.*, 1993). However, crystallographic determination of MHC structures with bound peptides has shown the peptides to be in extended, non-α-helical conformations, and so the physical or chemical basis for the observed α-helical side-chain periodicities was not apparent.

Two explanations for the source of the α-helical periodicities were presented by Cornette *et al.* (1995), working with the set of MHC binding motifs listed by Meister *et al.* (1995). The first explanation stems from the observation that the anchor residues fitting into the pockets of the MHC occur at spacings consistent with α-helical periodicity. Periodicity analysis was performed for the interaction between known MHC ligands and the MHC binding groove (Rammensee *et al.*, 1995). Spacing of the anchor residues together with the other preferred residues gives a hydrophobicity periodicity signal similar to that of helices (Cornette *et al.*, 1995). Thus, the periodicity of hydrophobicity of MHC-binding peptides may be due to the spacing of largely hydrophobic anchor residues in the peptide, enforced by the spacing of pockets in the MHC groove.

The second, mutually consistent source of near-α-helical periodicity in side-chain polarities in antigenic peptides stems from the observation that recent crystallographic determination of the class II HLA-DR1-bound peptide influenza haemagglutinin 307-319 (HA) (Stern *et al.*, 1994) showed the peptide to be in an extended conformation, but with a left-handed twist of about 130° per residue. The conformation is similar to the polyproline type II twist and is common in four- and five-residue structures in proteins, but is not common at lengths of 10 residues or

more. That twist, together with a separation of the side-chains with hydrophobic (hydrophilic) side-chains pointing into (or out of) the MHC cleft, induces a 2.8 residue per period repetition of side-chain polarities on the peptide. This could explain the observed periodicity of hydrophobicity without any α-helical structure. Consequently, structural constraints such as regularly spaced pockets or the twist that has been observed in several antigenic peptide backbones will cause a periodic variation in side-chain polarities (Cornette *et al.*, 1995). Thus the amphipathic helical signal continues to be an allele-independent indicator of potential antigenic sites in about two-thirds of known determinants presented by MHC class II molecules, and is highly significantly correlated with antigenicity.

An additional pattern-based algorithm was described by Stille *et al.* (1987): the strip-of-hydrophobic-helix algorithm (SOHHA). This algorithm calculates the mean hydrophobicity of an axial strip along a five-turn α-helix, and is similar in concept to the AMPHI algorithm. These pattern-based methods remain in use today.

Motif-based models for prediction of T-cell epitopes: Simple anchor-based motifs

Recent research into MHC-binding motifs that appear to enable successful binding of peptides to specific MHC molecules, has promoted the development of another approach to predictive algorithms. An MHC binding motif is a pattern that describes amino acid residues preferred for binding in the pockets of the MHC-binding groove. These pockets, eventually designated A, B, C, D, E and F, are located in the peptide-binding site formed by two α-helices and the β-pleated sheet of the MHC molecule. MHC-binding motifs were deduced by aligning sequences of peptides eluted from a given MHC or known to bind to a given MHC or sequencing of pools of peptides eluted from an MHC molecule, and identifying the strong signals of amino acids that were shared by most of the peptides at a given position (Falk *et al.*, 1991; Hobohm and Meyerhans, 1993; Rammensee *et al.*, 1995); the side-chains of these amino acids are presumed to interact in a favourable manner with amino acids lining the pockets of the MHC molecule's peptide binding groove. A list of peptides known to bind the HLA A*0201 and the deduced motif are illustrated in Fig. 3 and Table 1.

The occurrence of such motifs might identify some peptides that would bind to single MHC molecules. Following a similar line of thought, the location within a peptide of several MHC-binding motifs that correspond to a group of distinct MHC alleles, might effectively predict peptides with the capacity to bind to a variety of MHC molecules (promiscuous binders) and with the capacity to stimulate an immune response in these various MHC contexts as well (promiscuous epitopes) (Berzofsky *et al.*, 1991).

MHC-binding motifs for a wide variety of both human and murine MHC class I and class II alleles, as well as those specific to other species, have been published. However, it remains unclear whether such motifs hold great predictive value (for example, only about one-third of peptides containing the motif corresponding to a given MHC class I allele have

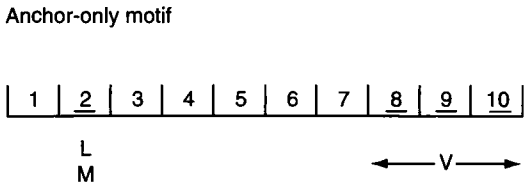

Figure 3. The anchor based motif, derived from aligning the peptides eluted from A*0201 and sequenced by Edman degradation (see Table 1) and identifying conserved residues at positions 2, 8, 9 and 10 (underlined).

Table 1. List of peptides that bind to A2

KLPQLCTEL	VLMEWLKTRPI	SMVGNWAKV
LLKYRAREPV	EMMTACQGV	DLHDSCSRNL
YMDDVVLGA	AIMDKNIIL	GLLGFVFTV
PLTVNENRRL	PIWKGPAKLL	TLQDIVLHL
GLGGGFGV	GLVGLRIVFA	DLSDGSWSTV
DLLMGTLGIV	VLPEKDSWTV	FMVFLQTHI
ILCWGELMTL	DLHVISNDV	ELQAIHLAL
WMELSVMEV	PLLYRLGAV	GLFGGGFGV
SLYNILSPFM	CLFHIVNLI	LLALLSCLTV
FLGPLLVLQA	GTLSKIFKL	TLAFDVWGQG
GLLGPLLVL	TIAPFGIFGTNY	YIGEVLVSV
TMDHARHGF	ILDISQGLS	MLWEGFTYI
GLGGGVGGV	LLDGTATLRL	ILGLLGRAV
GLPVCQDHL	GMNERPILT	GLSPTVWLSA
SLPTHDPSPL	TLTSCNTSV	RMPEAAPRV
QLRSLTEIL	VLIQRNPQL	VMNILLQYVV
EGPEYWDGETR	CLGWLTGMDI	GLFGGGGGL
PLGFFPDHQL	SLLPAIVEL	WLNEILWSI
RLSELEAALQRA	KALGFVFTL	YLLPAIVHI
HLPDRVHFA	KMADLVGFLV	TLPQEHIVL
MMWFVVLTV	RLRIVRGTQL	GLWIRTPPV
WLSLLVPFV	YLPDTLLLEECG	RLIVFPDLGV
FLLLADARV	ILKDPVHGV	AAPTPAAPA
PMGVGLSPFL	LLDPRVRGL	FLPSDYFPSV
CILESLFRA	PLDGEYFTL	KLWVTVYYGV
EILKEPVHGV	LLDTGADDTV	SLISDQLLM

been shown to be bound by that MHC molecule) (Calin-Laurens *et al.*, 1993; Pamer *et al.*, 1991; Lipford *et al.*, 1993; Nijman, 1993). A number of algorithms have been designed to identify peptides containing MHC-binding motifs within the peptide sequence. These algorithms are limited by the inherent imprecision of anchor-based motifs.

One such algorithm, EpiMer, attempts to account for this imprecision by selecting regions of proteins that contain clusters of MHC-binding potential, that may exhibit the properties of promiscuous binders or

promiscuous T-cell epitopes, with some success (Fig. 4) (Meister *et al.*, 1995; Roberts *et al.*, 1996). This algorithm uses a library of MHC-binding motifs for multiple MHC class I and class II alleles to predict antigenic regions within the protein sequence with the potential to induce an immune response in subjects with a variety of genetic backgrounds (Meister *et al.*, 1995; Roberts *et al.*, 1996). The approach is relatively simple. First, the algorithm locates matches to each MHC-binding motif in its database, along the length of a given protein antigen. Then, the relative density of these motif matches is determined along the length of the antigen, allowing the generation of a density histogram. Finally, EpiMer locates protein regions in this histogram with a motif match density above a selected density value, and produces a list of subsequences representing these motif-rich regions (Table 2). Such regions, due to their concentration of varied binding motif matches, may be more likely to act as promiscuous binding peptides than randomly chosen peptides from the same antigen. An example of a promiscuous epitope derived from *Clostridium tetanus* toxin, predicted by EpiMer and previously demonstrated to be a promiscuous epitope *in vitro*, is shown in Fig. 5 (Ho *et al.*, 1990).

The library of MHC-binding motifs used to locate such regions can be limited to as few as one motif, or as many as the total capacity of the current motif database, which is updated regularly from the literature. An example is given in Table 3; class II motifs are illustrated in this table. In this fashion, EpiMer can be tailored to search for different phenomena along the length of a protein antigen, depending on the information sought. For example, EpiMer can be instructed to seek regions that contain more than one MHC binding motif of a selected type. These regions, harbouring reiterative motifs (first characterized by Sette *et al.* (1990)) are likely to bind with high affinity to HLA-DR4 molecules, giving them a greater chance of inducing a cellular immune response.

One can also configure EpiMer to search for matches to MHC-binding motifs specific to a wide variety of MHC molecules, and use the resultant motif density histogram to pick out regions of protein antigens that contain an unusually high density of motifs for multiple alleles. The chosen peptide regions could act as promiscuous binders, binding to a variety of MHC alleles, owing to their inclusion of motifs corresponding to these alleles. Such peptides would be good candidates for synthetic peptide vaccines meant to elicit protective immunity in people with a variety of genetic backgrounds.

One methodological concern when designing a multiple binding motif-based predictive algorithm is the accuracy of the motifs used to predict putative epitopes, and thus the overall validity of the motif database. Often in the literature, previously reported motifs are redefined through peptide truncation and amino acid substitution experiments (Sidney *et al.*, 1992; Ruppert *et al.*, 1993); likewise, new emphasis has been placed on the identification of specific amino acid residues within peptides which interfere with their relative capacities to bind to the MHC groove (Boehncke *et al.*, 1993), and on the role of protein processing and presentation (Rammensee *et al.*, 1995).

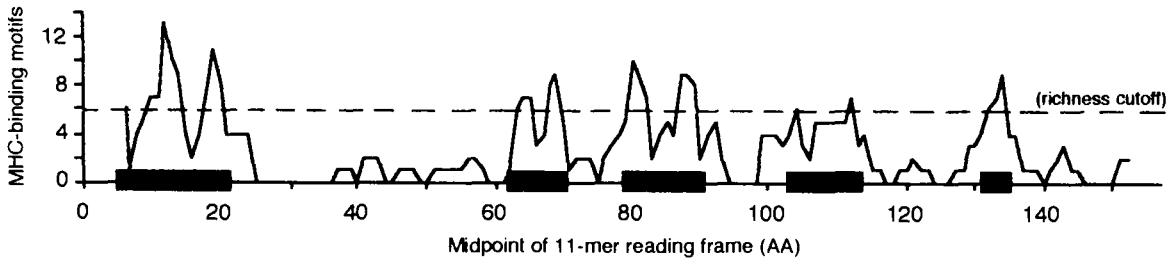

Figure 4. The density of MHC-binding motif matches along the sequence of the 19-kDa protein of *M. tuberculosis* is shown here to illustrate the EpiMer method of putative epitope identification. For this analysis, only human class II MHC-binding motifs were used in the search. Peptides that include peaks of motif density, such as the 10- to 25-mers including amino acids as shown by the black bars on the *x* axis in this example are predicted as putative T-cell epitopes by the EpiMer algorithm.

Table 2. EpiMer output: regions of the 19-kDa Mtb protein containing MHC class II binding motifs

Sequence	Start	Stop	Allele
KITGTATGV	130	138	HLA-DP9
VKRGLTVAV	1	9	HLA-DPA1*0102/DPB1*0201
MANPMSPVN	140	148	HLA-DPA1*0102/DPB1*0201
VKRGLTVAVA	1	10	HLA-DPw4(b)
VAVAGAAILV	7	16	HLA-DPw4(b)
AVAGAAILVA	8	17	HLA-DPw4(b)
AGAAILVAGL	10	19	HLA-DPw4(b)
VVCTTAAGNV	64	73	HLA-DPw4(b)
IAIGGAATGI	75	84	HLA-DPw4(b)
YKITGTATGV	129	138	HLA-DPw4(b)
VDMANPMSPV	138	147	HLA-DPw4(b)
GLTVAVAGA	4	12	HLA-DQ3.1
TVAVAGAAI	6	14	HLA-DQ3.1
AVAGAAILV	8	16	HLA-DQ3.1
VAGAAILVA	9	17	HLA-DQ3.1
AGAAILVAG	10	18	HLA-DQ3.1
GAAILVAGL	11	19	HLA-DQ3.1
LVAGLSGCS	15	23	HLA-DQ3.1
TTTAAGTTA	34	42	HLA-DQ3.1
AGTTASPGA	38	46	HLA-DQ3.1
SPGAASGPK	43	51	HLA-DQ3.1
VTGSVVCTT	60	68	HLA-DQ3.1
TGSVVCTTA	61	69	HLA-DQ3.1
VCTTAAGNV	65	73	HLA-DQ3.1
CTTAAGNVN	66	74	HLA-DQ3.1
AAGNVNIAI	69	77	HLA-DQ3.1
AIGGAATGI	76	84	HLA-DQ3.1
IGGAATGIA	77	85	HLA-DQ3.1
AATGIAAVL	80	88	HLA-DQ3.1
ATGIAAVLT	81	89	HLA-DQ3.1
GIAAVLTDG	83	91	HLA-DQ3.1
IAAVLTDGN	84	92	HLA-DQ3.1
GLGNVNGVT	100	108	HLA-DQ3.1
GQGNASATK	116	124	HLA-DQ3.1
GNASATKDG	118	126	HLA-DQ3.1
ITGTATGVD	131	139	HLA-DQ3.1
TATGVDMAN	134	142	HLA-DQ3.1
VKRGLTVAV	1	9	HLA-DQ7
VKRGLTVAV	1	9	HLA-DR1(c)
LTVAVAGAA	5	13	HLA-DR1(c)
VAVAGAAIL	7	15	HLA-DR1(c)
AVAGAAILV	8	16	HLA-DR1(c)
VAGAAILVA	9	17	HLA-DR1(c)
ILVAGLSGC	14	22	HLA-DR1(c)

Table 2. *contd.*

Sequence	Start	Stop	Allele
AGTTASPGA	38	46	HLA-DR1(c)
VCTTAAGNV	65	73	HLA-DR1(c)
AIGGAATGI	76	84	HLA-DR1(c)
IGGAATGIA	77	85	HLA-DR1(c)
AATGIAAVL	80	88	HLA-DR1(c)
LTDGNPPEV	88	96	HLA-DR1(c)
VKSVGLGNV	96	104	HLA-DR1(c)
VGLGNVNGV	99	107	HLA-DR1(c)
LTVAVAGAA	5	13	HLA-DRB1*0101(R)
VAVAGAAIL	7	15	HLA-DRB1*0101(R)
VNIAIGGAA	73	81	HLA-DRB1*0101(R)
IGGAATGIA	77	85	HLA-DRB1*0101(R)
IAAVLTDGN	84	92	HLA-DRB1*0101(R)
LGNVNGVTL	101	109	HLA-DRB1*0101(R)
GLTVAVAGA	4	12	HLA-DRB1*0401(DR4Dw4)(a)
VAGAAILVA	9	17	HLA-DRB1*0401(DR4Dw4)(a)
AGAAILVAG	10	18	HLA-DRB1*0401(DR4Dw4)(a)
GAAILVAGL	11	19	HLA-DRB1*0401(DR4Dw4)(a)
AGNVNIAIG	70	78	HLA-DRB1*0401(DR4Dw4)(a)
ATKDGSHYK	122	130	HLA-DRB1*0401(DR4Dw4)(a)
GVDMANPMS	137	145	HLA-DRB1*0401(DR4Dw4)(a)
LSGCSSNKS	19	27	HLA-DRB1*0402(DR4Dw10)
VDGKDQNVT	53	61	HLA-DRB1*0402(DR4Dw10)
MANPMSPVN	140	148	HLA-DRB1*0402(DR4Dw10)
VKRGLTVAV	1	9	HLA-DRB1*0404(DR4Dw14)
LSGCSSNKS	19	27	HLA-DRB1*0404(DR4Dw14)

Motif-based models for prediction of T-cell epitopes: Extended anchor motifs

One method for addressing the inherent inaccuracy of anchor-based motifs has been to examine lists of peptides known to bind to a given MHC for additional characteristics, such as 'favoured' or auxiliary residues, and residues that appear to inhibit binding (Fig. 6) (Ruppert *et al.*, 1993). These methods are computationally more complex than straightforward anchors, and may or may not significantly improve the accuracy of peptide predictions.

Rammensee *et al.* (1995) also proposed a similar tactic, listing certain amino acid residues that appeared to be required and others that were preferred, basing their assumptions on an analysis of actual MHC-binding peptides and known T-cell epitopes. Parker *et al.* (1994) have derived an algorithm from these descriptions, based on anchors and preferred or inhibitory residues described in the literature, in which binding of a putative ligand to an MHC molecule is predicted by a function of the residues at each position. This algorithm is available for use predicting T-cell epitopes

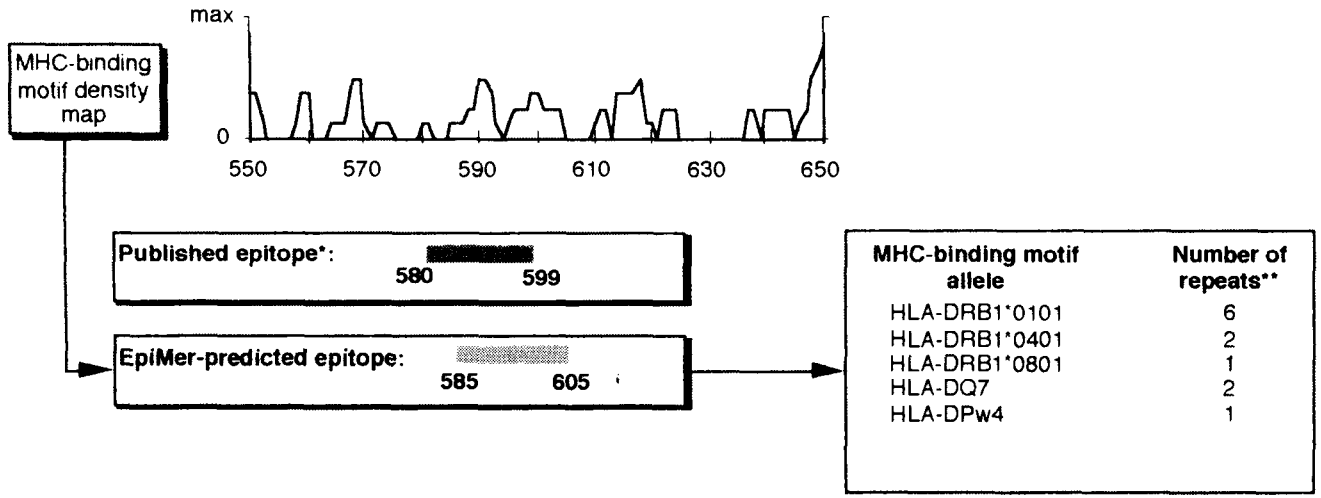

Figure 5. Clustering of T-cell epitopes. Both the published 'promiscuous' epitope and the EpiMer prediction contain multiple MHC class II binding motifs.

Table 3. Class II anchor-based MHC-binding motifs compiled from the literature

Motif name	Reference(s)	Position in peptide									
		i	*i* + 1	*i* + 2	*i* + 3	*i* + 4	*i* + 5	*i* + 6	*i* + 7	*i* + 8	*i* + 9
2.HLA-DP9	Dong *et al.* (1995)	R, K					A, G, L			L, V	
2.HLA-DPA1*0102/DPB1*0201	Rammensee *et al.* (1995)	F, L, M, V, W, Y				F, L, M, Y			I, A, M, V		
2.HLA-DPw4(b)	Falk *et al.* (1994)	F, L, Y, M, I, V, A						F, L, Y, M, V, I, A			V, Y, I, A, L
2.HLA-DQ2	Verreck *et al.* (1994)	K			I					F	
2.HLA-DQ3.1	Sidney *et al.* (1994)	no R, K, D, E, P	no R, K, D, E	A, G, S, T	no D, E	A, V, L, I					
2.HLA-DQ7	Falk *et al.* (1994)	F, Y, I, M, L, V				V, L, I, M, Y		Y, F, M, L, V, I			
2.HLA-DR1(a)	Hammer *et al.* (1992)	Y, F, W	no D, E	no D, E	M, L	no D, E	G, A	no D, E	no D, E	L, M, A, I, G, T, V, Q, S	
2.HLA-DR1(c)	Kropshofer *et al.* (1992)	A, V, I, L, Y, F, W, M, C					S, T, A, V, I, L, P, C			A, V, I, L, Y, F, W, M, C	
2.HLA-DR1(d)	Hammer *et al.* (1994b)	Y, W, F, I, L, V, M	R		M, L	G, A			L		
2.HLA-DR3(b)	Sidney *et al.* (1992)	A, V, I, L, Y, F, W, M, C		A, V, I, L, Y, F, W, M, C	Q, N, R, K, D, E, S, T		R, K, H				
2.HLA-DRB1*0101(R)	Rammensee *et al.* (1995)	Y, V, L, F, I, A, M, W			L, A, I, V, M, N, Q		A, G, S, T, P			L, A, I, V, N, F, Y, M	
2.HLA-DRB1*0301	Chicz *et al.* (1993a)	Y, F, W, L, I, V, M			D, E, N					Y, M, L, I	
2.HLA-DRB1*0301(a)	Malcherek *et al.* (1994) Stretch variant of (41) Rammensee *et al.* (1995)	L, I, F, M, V			D		K, R, E, Q, N		L, Y, F		
2.HLA-DRB1*0301(b)	Malcherek *et al.* (1994) Also (41) Rammensee *et al.* (1995)	L, I, F, M, V			D		K, R, E, Q, N			L, Y, F	
2.HLA-DRB1*0301(c)	Malcherek *et al.* (1994) Stretch variant of Rammensee *et al.* (1995)	L, I, F, M, V			D		K, R, E, Q, N				L, Y, F
2.HLA-DRB1*0401(DR4Dw4)(a)	Rammensee *et al.* (1995)	F, Y, W, I, L, V, M, G, A			F, W, I, L, V, A,		N, S, T, Q, H, R, V, L, I, M D, E, M		D, A, S, V, H, P, L, N, M, I	A, S, Q, G, L, T, V, K	
2.HLA-DRB1*0402(DR4Dw10)	Rammensee *et al.* (1995)	V, I, L, M			no D, E		N, Q, S, T, K	R, K, H, N, Q, P		A, H, G, Q, S, N, L, T, V	
2.HLA-DRB1*0404(DR4Dw14)	Rammensee *et al.* (1995)	V, I, L, M			no R, K		N, T, S, Q, R	A, N, V, Q, K, P, D, M, S, H, L, I, T		A, S, Q, G, L, T, V, K	
2.HLA-DRHB1*0405(DR4Dw15)(a)	Rammensee *et al.* (1995)	F, Y, W, V, I, L, M			V, I, L, M, D, E		N, T, S, Q, K, D, V	A, N, V, Q, K, P, D, M, S, H, L, I, T		D, E, Q	
2.HLA-DRB1*0701	Chicz *et al.* (1993a)	W, Y, F, M, V, L, I					T, S			W, Y, F, M, V, I	
2.HLA-DRB1*0801	Chicz *et al.* (1993a)	Y, F, M, V, L, I				K, R					
2.HLA-DRB1*1101	Rammensee *et al.* (1995)	W, Y, F			M, L, I, V		R, K				
2.HLA-DRB1*1201	Rammensee *et al.* (1995)	I, L, F, Y, V		L, M, N, V, A			V, Y, F, I, N, A			Y, F, M, I, V	
2.HLA-DRB1*1501	Chicz *et al.* (1993a)	L, I, V			Y, F, W, I, V			F, L, I, V, M			
2.HLA-DRB5*0101	Rammensee *et al.* (1995)	F, Y, L, M			Q, V, I, M					R, K	
2.HLA-DRB5*0101(a)	Chicz *et al.* (1993a) Adjusted based on (41) Rammensee *et al.* (1995)	Y, F, L, M			V, M, I, Q				K, R		

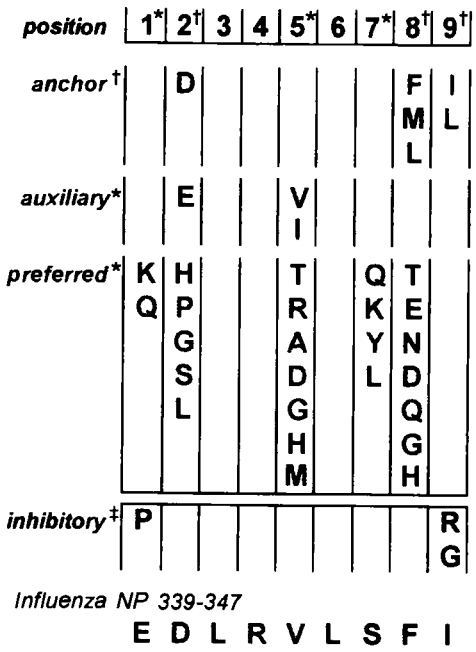

Figure 6. The extended binding motif for HLA B*3701 (from Rammensee *et al.*, 1995). The sample B*3701 binding peptide from influenza nucleoprotein (amino acids 339–347) contains a primary anchor (D at position 2), an auxiliary anchor V at position 5, another primary anchor F at position 8 and I at position 9.

on the BIMAS website (http://bimas.dcrt.nih.gov/molbio.hla_bind/) (Parker *et al.*, 1994). The BIMAS site also incorporates a prediction method based on information derived from competitive binding assays (see the description of the Parker matrix method, below).

Altuvia *et al.* (1994) have developed an automated computerized method that enables the identification of common motifs in sets of peptides that are known to bind to a certain MHC molecule by incorporating information about peptides known to bind as well as those known *not* to bind. This method identifies common motifs in unaligned sequences; in other words, the common motif does not necessarily reside in a unique location in the different sequences, and it identifies motifs that are based on physico-chemical and structural properties that are sequence dependent and may play a role in specific recognition (e.g. hydrophobicity, charge, hydrogen bonding potential). These two properties of the algorithm are especially important for the identification of common motifs in peptides presented by class II molecules, which show a large variation in sequence and length.

The algorithm compiles, for each class II molecule, a database that includes two groups of peptides: those that bind to that MHC molecule to elicit a T-cell response and those that have been shown experimentally

either not to bind to that MHC molecule or to fail to elicit a T-cell response. The second step of the algorithm is analysis of the aligned peptide sequences for additional, less conserved, sequence-dependent features in positions other than those that define the motif. This algorithm has been demonstrated on a number of MHC molecules and their related peptides. The identified motifs were found to be consistent with experimental results of substitution experiments and with the solved crystal structure of the DR1 molecule complexed with an influenza haemagglutinin peptide (Altuvia *et al.*, 1994).

Motif-based models for prediction of T-cell epitopes: Supermotifs

Another approach to the identification of MHC ligands and T-cell epitopes has been to determine whether the peptides that bind to selected MHC molecules share a set of determinants. Conservation of the amino acid residues lining the pockets in the peptide binding groove of the MHC may contribute to the development of MHC superfamilies or supertypes. Sidney *et al.* (1996a) propose to exploit these groupings of HLA types by the residues located in the binding pocket. This might simplify the search for T-cell epitopes: rather than searching for a single peptide motif for each HLA allele, such 'supermotifs' would permit the identification of epitopes capable of binding to multiple HLA types within the supertype. For example, selected peptides were noted to be capable of binding to both A*0301 and A*1101 with high affinities (Kast *et al.*, 1994). Sidney and co-workers have designated a number of alleles as belonging to the A3 superfamily: A*0301, A*1101, A*3101, A*3301, A*3401, A*6601, A*6801, and A*7401 (Sidney *et al.*, 1996b). B7, A2, and B17 superfamilies have also been described (Sidney *et al.*, 1995; Barber *et al.*, 1997; del Guercio *et al.*, 1995). Supermotifs for class II processing (Kropshofer *et al.*, 1993) have been proposed, as have supermotifs that may govern the multideterminant binding of CLIP (Malcherek *et al.*, 1995; Sinigaglia and Hammer, 1995).

Motif-based models for prediction of T-cell epitopes: Matrix-based motifs

Matrix-based motifs have been developed to improve on the specificity of anchor-based motifs: the advantage of matrix motifs is that peptides can be given a score that represents the sum of the potential for each amino acid in the sequence to promote or inhibit binding.

Sidney *et al.* (1996a) have derived a matrix-based algorithm from a method known as 'peptide side-chain scanning', which they used to predict binding peptides for the MHC allele DRB1*0401. The group chose a suitable 'blank' binding peptide (an alanine-substituted analogue of a good DRB1*0401 binder), and proceeded to determine the effects on binding of single amino acid substitutions in each position along this peptide (Fig. 7). This allowed the construction of a database of all possible amino acid side-chain effects for a single MHC-binding motif, which was later converted into an algorithm able to run through a protein's primary structure and to predict, within reasonable error, the binding capacities of all

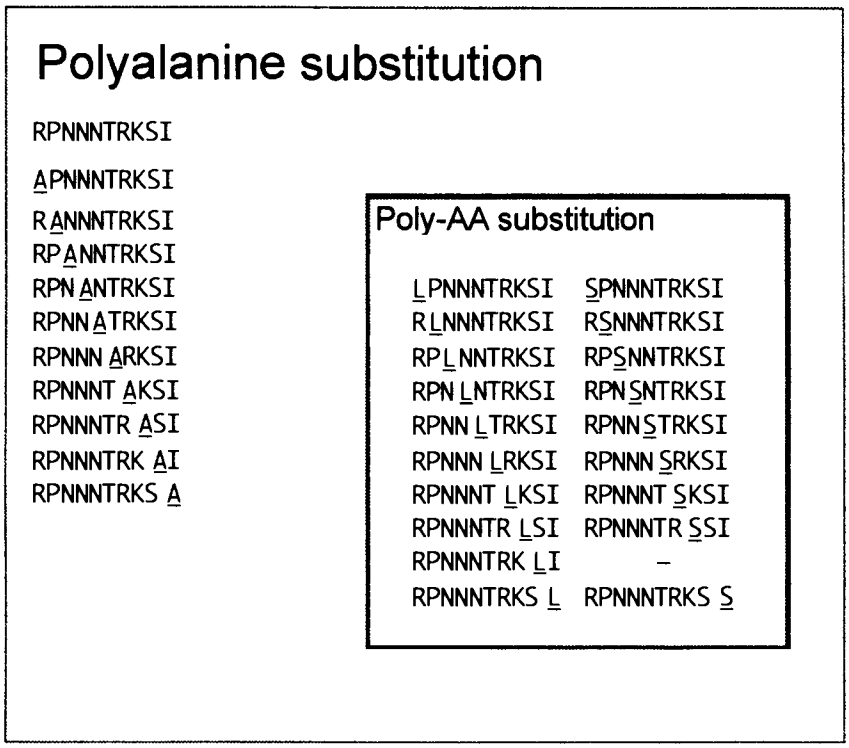

Figure 7. Identification of anchor positions can be estimated by substituting each amino acid in a peptide with alanine, to see which residues are crucial for binding (or, alternatively, recognition). A fuller picture of the subtle influences of different amino acids at each position can be generated by substituting several different (or even all naturally occurring) amino acids and measuring the relative binding of each variant peptide.

possible peptides of a fixed length to a single MHC molecule. Marshall *et al.* (1995) have designed a nearly identical algorithm, and have taken this method a step further by showing some preliminary results that implicate a high correlation of predicted binding affinity with immunogenicity. Parker *et al.* (1994) have described the development of quantitative matrices based on dissociation half-life of MHC–peptide complexes; an algorithm based on these matrices is available on the BIMAS website (http://bimas.dcrt.nih.gov/molbio.hla_bind/). Davenport *et al.* (1995, 1997) have also developed a matrix-based method for MHC-ligand prediction using data derived from Edman degradation of pooled elutions from MHC molecules.

Each group's technique is based on the assumptions of independence of each position and a sequence-independent peptide conformation, where side-chain substitutions have little, if any, effect on the conformational characteristics of an MHC-bound peptide, but rather exert their influence on the peptide's binding strength. These assumptions are sup-

ported in the literature (Hammer *et al.*, 1994a; Stern *et al.*, 1994), but must represent a first approximation to which exceptions may be found.

Motifs for class II molecules have been more difficult to derive, primarily because eluted peptides may be of several different lengths and therefore difficult to align. Class II matrices have been derived by Fleckenstein *et al.* (1996), who have developed synthetic undecapeptide libraries (11 amino acid long peptides substituted at every position with one residue held constant), performed competitive-binding assays, and described the contribution of every potential residue at each binding position. These data were used to derive an algorithm that accurately predicted the binding of a set of peptides designed to maximize binding potential (Jesdale *et al.*, 1997).

Matrix-based MHC ligand prediction: EpiMatrix

The TB/HIV Research laboratory team has constructed matrix motifs for 30 HLA class I alleles from lists of peptides known to bind to a given MHC, and from information on peptides eluted from MHC and published in the literature. The matrix motifs serve as patterns to which potential binding regions from primary protein sequences are compared (Plate 1). Each putative MHC-binding region within any given protein sequence is scored according to its fit to the matrix motif; higher EpiMatrix scores are expected to indicate greater MHC binding potential (Jesdale *et al.*, 1997).

Table 4. Prediction of published HLA B7 ligands utilizing EpiMatrix v 11.1.96

HLA-B7

Name of Protein	Known ligand sequence	Protein length	EpiMatrix rank of ligand
Topoisomerase II	SPRYIFTML	1621	1
EBNA 3A	RPPIFIRRL	812	1
HLA-A2.1 signal sequence	APRTLVLLL	365	1
HLA-DP signal sequence	APRTVALTAL	258	1
Ribosomal S26 protein	APAPPPKPM	107	1
HLA-B7 signal sequence	LVMAPRTVL	255	2
HIV V3	RPNNNTRKRI	90	2
Histone H1	AASKERSGVS L	219	7
EBNA 3C	APIRPIPTRF	983	21

Known HLA B7 restricted ligands are shown for each of nine proteins. EpiMatrix was applied to the sequences of these proteins; 10-mers overlapping by nine proteins were scored for each of the original protein sequences. The rank of the peptide that corresponded to the known ligand is listed in the final column. In the case of topoisomerase II, for example, 1612 10-mers were evaluated and ranked by EpiMatrix score. The 10-mer that was scored the highest by EpiMatrix was identical in sequence to the published ligand for this protein. The published ligand corresponded to the highest ranked Epimatrix prediction for the same protein in four of the remaining eight cases. A total of 4620 10-mers were scored and ranked by EpiMatrix for this analysis; in seven of nine cases the ligand would have been correctly identified had only the two top-scoring EpiMatrix peptides been synthesized.

Comparisons between EpiMatrix predictions and ligands published by Rammensee *et al.* (1995) can be summarized as follows: Over 60 000 potential ligands for these 133 proteins were scored and ranked. For the 133 proteins evaluated, 85% of known ligands would have been identified if only the top 10 scored EpiMatrix predictions for these proteins had been synthesized and tested. The number of correctly identified published ligands would have increased to 95% if the top 20 peptides had been synthesized and tested. An example of a comparison between EpiMatrix predictions and ligands reported by Rammensee *et al.* (1995) is given in Table 4.

As the contributions of side chains to peptide–MHC binding become better quantified, the development of a computer algorithm that predicts T-cell epitopes based on a matrix of quantitative motifs, such as the ones described by the TB/HIV Research Laboratory (Meister *et al.*, 1995; Roberts *et al.*, 1996; Jesdale *et al.*, 1997), Davenport (1995), Fleckenstein *et al.* (1996), Hammer (1995) and Parker *et al.* (1995) will only be a matter of time.

Estimation of peptide binding based on prediction of structures of MHC class I–peptide complexes

The homologous extension method is the most straightforward computational approach to determining the structure of peptides bound to MHC class I molecules. The most closely related MHC–peptide complex with a known crystal structure is used as a model, and all the positions at which the sequences differ are replaced with the amino acids of the target sequence. The clashes between peptide and MHC side-chains created by these substitutions are removed by altering the conformations of the peptide and MHC side-chains until a minimum energy is found. The process of varying the conformations of the substituted side-chains, evaluating the energies of the generated structures, and determining the lowest-energy structure is usually carried out using a standard software package, such as CHARMm. Using cases for which a crystal structure is known to test the procedure, the average error in the predicted backbone atom co-ordinates (root-mean-square deviation (RMSD) from the known crystal structure) is typically < 0.2 nm. However, large side-chain deviations and all atom RMSDs frequently occur, and each step can contribute to misleading results.

Of course, varying only side-chain conformations is too limiting and is not realistic. Even among peptides binding the same MHC, the central α carbon atoms can deviate by > 0.2 nm, and the directions of the Cα–Cβ bonds at corresponding locations can change, imposing substantial variation in the orientation of side-chains. The situation is even worse if the crystal structure of the MHC of interest is not known, when the RMSD of corresponding α-carbon atom co-ordinates may be > 0.4 nm, with the corresponding side-chains sometimes pointing in almost opposite directions. Another major problem is the need to use free energy rather than energy, since energy alone cannot effectively find the most stable orientation of a side-chain, even if all orientations are examined, and cannot correctly rank order different side-chains at the same position. For example, although the

experimentally determined best anchor at position two in peptides bound to HLA-A2.1 is a hydrophobic Leu, application of the procedure using the standard CHARMm energy predicts the most stable side-chain at position 2 to be a charged Lys (Engelhard, 1994). However, carrying out the procedure with a recently developed free energy model (Vajda *et al.*, 1994) correctly predicts the Leu anchor (Madden *et al.*, 1992). Importantly, this model provides a rapid procedure for evaluating the solvation free energy.

A promising approach for computational determination of anchors of uncrystallized class I–peptide complexes would start by constructing a free-energy map of the binding site. As a first approximation, mapping is local. This simplified approach is useful for docking and for predicting how stability is affected by side-chain substitutions. At a given position, the amino acid (C_α) is translated over a small volume surrounding the corresponding C_α position in a peptide in a homologous MHC of known crystal structure. At each position an orientational search is performed, and then at each orientation a conformational search is carried out. Since the lowest free energy for each amino acid will not necessarily give the lowest overall free energy for the whole peptide, for each amino acid both the best free energy and some slightly higher free energy states are determined. These are concatenated to obtain possible structures for the peptide, which are then ranked according to their overall free energies. The concatenation process can be carried out using a number of procedures, such as dynamic programming (Vajda and DeLisi, 1990; Gulukota *et al.*, unpublished).

To test this type of approach starting with the highly non-homologous histone-HLA B27 structure (Fremont *et al.*, 1992; Madden *et al.*, 1992), Sezerman *et al.* (1996) successfully predicted a structure for the known SEV9 peptide bound to H-2K^b, with an all-atom RMSD from the crystal structure of 0.12 nm. In the reverse direction, the HLA B27-bound histone peptide was predicted from the SEV9/K^b complex to within 0.15 nm. The HTLV-1, HIV-1 RT and influenza matrix protein peptides bound to HLA-A2.1 were also predicted to within 0.14, 0.13 and 0.16 nm all-atom RMSDs, respectively, in each case starting with the HLA-A2.1-bound HIV-1-GP peptide (Madden *et al.*, 1992).

Given the accuracy of free energy predictions reported in the test cases (Sezerman *et al.*, 1996), it may be possible to develop simple predictive methods for identifying MHC binding peptides both (1) by extending existing motifs for MHC molecules for which only key anchor residues are known, by incorporating the effects of each possible amino acid at other positions in the peptide, and (2) by predicting new binding motifs for MHC alleles for which motifs are not known. By expanding the number of alleles for which appropriate vaccine peptides can be made, greater coverage of the HLA diverse population can be accomplished (Gulukota *et al.*, unpublished).

Prediction of peptide–MHC binding by a threading approach

Altuvia *et al.* (1995) have developed a novel application of the threading concept to estimate the binding energies of peptides in the MHC groove.

The threading method, developed to address the problem of protein folding, uses a library of known folds found in proteins. Each structure is treated as a chain racing through space, ignoring the original amino acid sequence itself, to create a structural template. The sequence being analysed is threaded through each of these templates and its energy is evaluated by empirical pairwise contact potentials. The lowest-energy threading path is taken as predictive of the native structure.

Altuvia *et al.* (1995) reasoned that this approach could be applied to prediction of peptide binding to MHC based on the crystallographic observation by Madden *et al.* (1994), that four different 9-mers and one 10-mer bind very similarly in the HLA-A2 groove. Thus, they could use the spatial path of the peptides in the groove as a template for threading of other peptides. The binding energies could be evaluated by empirical contact potentials between residues in the peptide and in the MHC molecule that are in contact in the complex. Altuvia *et al.* developed an algorithm that maps the amino acids of a peptide sequence to the crystallographic co-ordinates of the backbone fold of a peptide in a solved MHC–peptide crystal structure. Most of the amino acids in the peptide interact with neighbouring MHC residues. The net interaction energy of a given peptide residue is calculated as the sum of its interaction energies with all of the MHC residues in close contact, and that of the whole peptide is the sum for all the residues. The predicted binding energies to a given MHC molecule of overlapping peptides covering a protein sequence can thus be ranked.

To test this threading approach, Altuvia *et al.* (1995) showed that known antigenic peptides are highly ranked among all possible peptides in a given protein sequence. This was true for the five peptides crystallized in HLA-A2 as well as other known peptides presented by HLA-A2. The approach also succeeded in predicting the binding hierarchy of peptides ranked by competition binding experiments, including a large number of 9-mers and 10-mers for which experimental binding data were available. The advantage of this threading algorithm for ranking potential binders to a given MHC molecule is that it is independent of binding motifs, and can identify good binders lacking known motifs. It can also be used to rank known motif-containing peptides according to their predicted binding energies. The algorithm is easy to apply and runs quickly. Its major limitation, that it requires a three-dimensional structure of at least one peptide in the same MHC molecule, may be overcome by modelling other MHC molecules based on the crystal structure of those already determined.

Artificial neural networks

Artificial neural networks (ANNs), such as those implemented by Brusic *et al.* (1994), Gulukota *et al.* (1997) and others have been used to identify peptides predicted to bind to selected MHC alleles. Given a database of peptides that are known to bind to a given MHC molecule such as those available through the MHCPEP website (Brusic *et al.*,

1996), ANNs can be trained to identify patterns that are common to peptides that bind. Recently, Ramakrishna *et al.* (unpublished) described the use of ANNs to predict peptides that may bind to A1 and A2.1, and compared their predictions with those published in the literature with good results.

◆◆◆◆◆◆ ADDITIONAL CONSIDERATIONS

Most of the models discussed in the preceding section do not account for peptide processing and transport. Motifs that predict proteolytic cleavage sites and characteristics of peptides that are likely to be transported into the ER remain to be described fully. These additional determinants may significantly improve predictive methods. Experimental methods such as tandem mass spectrophotometry, as described by Hunt *et al.* (1992) appear to be the best approaches to the determination of T-cell epitopes from unknown antigens. Predictive methods such as EpiMatrix, which currently provides predictions for greater than 30 different MHC alleles, may prove to be more cost-effective for antigenic proteins for which the full sequence is available (but are unable to account for post-processing modifications).

The methods for T-cell epitope identification described here may contribute to the identification of T-cell epitopes. However, most of the methods suffer from an imbalance between sensitivity and specificity. The experimentalist, who wishes to ascertain the entire repertoire of potential T-cell epitopes derived from an antigenic protein should select a method approximating the overlapping peptide method. In contrast, selected epitope prediction models may prove more cost and labour conservative for vaccine design problems in which only a subset of epitopes need be identified. The accuracy of these predictive methods needs to be established better *in vitro*. More specifically, the prediction of MHC binding potential, whether determined computationally or experimentally, is a useful screen, but should not be the sole determinant of T-cell epitope selection.

An overview of these approaches would seem to suggest that development of more accurate T-cell epitope prediction schemes, based on multiple features correlated with both antigenicity and high peptide binding affinity, is within reach. The future of T-cell epitope prediction may depend on the design of a suitable 'meta-algorithm' allowing the location of multiple features which have been demonstrated independently to predict immunogenicity or high binding affinity. A hypothetical algorithm may employ the subalgorithms such as EpiMatrix, or the algorithms of Vadja *et al.*, Hammer *et al.*, Marshall *et al.* and Altuvia *et al.* to predict the precise prediction of single-allele-binding peptides, implemented in an EpiMer-like multiple motif-based prediction model. Such a model could locate regions of a protein which contain 'clusters' of independently high binding affinity for multiple alleles. This model could also include the search for potentially amphipathic structures, and for peptides that are

predicted to be transported to the ER by TAP (Van Endert *et al.*, 1995) and compare these structures with regions of 'clustered' potential binding affinity, to select the peptides most promising to test, i.e. peptides with an optimal combination of features. Such an 'optimal combination' may even be determined by the nature of the pathogen in question, or by the vaccination strategy.

Importantly, the models described above are not mutually exclusive. In fact, the identification of novel structural features which are able to predict peptide binding or immunogenicity independently and their subsequent synthesis into a robust algorithm with statistically verifiable predictive capacity, will allow the discovery of more subtle sub- or superfeatures, which will give further insight into the nature of antigenicity (such as the clustering of motifs described by EpiMer). A suitable T-cell epitope prediction algorithm would allow for a dramatic reduction in the time and effort required to synthesize and test potential T-cell antigenic sites, by allowing the prediction of sites with a high concentration of antigenic features. Newly discovered features could then be implemented as part of the same algorithm, to hone its predictive capacity further, and further the rational selection of peptides aimed at the generation of a cellular immune response.

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PART II

Murine Models

◆◆

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1 Management of Immunocompromised and Infected Animals

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INTRODUCTION

The field of immunology has increasingly developed over the last decades and now requires a wide range of methods. Beginning with the application of attenuated infectious agents for vaccine production in integer animals, the majority of experiments today are performed *in vitro*, (e.g. phagocytosis, cytotoxic activity, signal transduction). However, to cover the complexity of the immune system, additional *in vivo* experiments are indispensable.

In the last decade naturally occurring and artificially induced immunodeficient animals have been widely used to study different aspects of immunity (Viney, 1994), such as autoimmunity (Benoist and Mathis, 1993), T-cell receptor repertoire (Mittruecker *et al.*, 1995), B cell compartment (Gu *et al.*, 1991), effects of adhesins (Mayadas *et al.*, 1993), functions of cytokines (Kopf *et al.*, 1995a,b; Trinchieri, 1997) and pathogenesis of

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infections (Kaufmann and Ladel, 1994). From these studies, the high complexity and considerable redundancy of the immune system became evident. In the context of managing immunodeficient animals, the finding of a spontaneously developing chronic ulcerative enterocolitis (inflammatory bowel disease) in a T-cell receptor mutant (Mombaerts *et al.*, 1993), Il-2 (Sadlack *et al.*, 1993; Mähler *et al.*, 1996) and Il-10 deficient mice (Kühn *et al.*, 1993) is of interest.

A large number of the transgenic mutants used in particular in the field of immunology are immunodeficient, being more or less susceptible to infections. Therefore, optimal hygienic standards are indispensable for these animals. We will try to point out here the special requirements for the management, breeding and housing of immunocompromised and infected animals, especially those for the mouse and rat.

◆◆◆◆◆◆ MICROBIOLOGICAL STANDARDIZATION

The quality of laboratory animals, mainly rodents, has improved during the last decade. The first attempts at eliminating disease were made in the 1950s. At that time infectious agents were widespread in rodent colonies, and many experiments were interrupted by infections. It became obvious that classical veterinary approaches, such as improved husbandry, vaccination, antibiotics and chemotherapeutics, would not eliminate pathogens, and therefore gnotobiotic techniques such as caesarean derivation and subsequent raising in isolation were established. This resulted in the elimination of various organisms, such as *Mycoplasma pulmonis*, which had previously been ineradicable. However, infections were still prevalent in many colonies. More sophisticated experimental procedures were increasingly sensitive to the influence of viruses. Some viruses had been tolerated in the past as they have a low potential to induce clinical disease, but both scientists and breeders were aware of their presence. It was shown later that many of these agents, although clinically silent, can induce increased variation between individuals and can influence biochemical or immunological functions. Research complications occurred frequently, resulting in the need to eliminate also those agents that cause clinically silent infections, and to monitor colonies of rodents for the presence or absence of such organisms.

Today, it is generally accepted that good research requires animals that are free from micro-organisms that might influence the health of the animals (or humans) or the results of experiments.

Influence of Micro-organisms on Research Results

It is generally accepted that research complications due to overt infectious diseases are significant and that clinically ill animals should not be used for scientific experiments. The effect of clinically silent infections, however, may be devastating, because they often remain undetected. Scientists in general are not well informed of such influences on their research. Only a small percentage of detected complications has been

published. The literature is scattered across diverse scientific journals, and many articles are difficult to locate. To address this problem, conferences have been held on viral complications on research, and the knowledge available summarized in conference proceedings (Bhatt *et al.*, 1986a; Hamm, 1986). The problem has been reviewed by Lussier (1988), the National Research Council (1991) and Hansen (1994).

Research complications may occur in various ways. Although acute clinical signs may not be observed, infected animals may show altered behaviour, suppressed body weight, or reduced life-expectancy, which may, for example, influence the tumour rate. Micro-organisms present in an animal may lead to contamination of samples and tissue specimens such as cells, tumours, sera and monoclonal antibodies. This may interfere with experiments performed with cells or isolated organs.

The experiment itself may be a stress factor and increase the sensitivity to an agent, and thus induce clinical disease or death. Environmental factors, such as increased temperature or relative humidity (for example, in metabolic cages), may induce stress which activates latent infections resulting in lung complications caused by *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus* or *Pneumocystis carinii*, especially in immunodeficient animals. Naturally, various micro-organisms can interact and lead to clinical disease or research complications, which are dependent on the combination of micro-organisms.

The disease rate is not only dependent on the host, but also on specific properties of the infectious agents. There are different strains of many viruses, with different organotropism (e.g. hepatotropic, enterotropic and neurotropic strains of the mouse hepatitis virus (MHV)). This influences the disease rate and the mortality, as well as the type and severity of pathological changes. For example, the immunosuppressive variant of the minute virus of mice (MVMi) replicates in lymphocytes, whereas the prototype strain (MVMp) replicates in fibroblasts, thus resulting in different effects on animals or experiments. Both variants usually do not induce clinical disease, but may affect various parameters such as wound healing, immunological reactivity, tumour growth and development, embryonic development and birth rate.

Various effects are possible on the function or the morphology of organs or cell systems. Histopathological changes that resemble adenomas have been observed in the trachea or bronchioles during the regenerative phase after a Sendai virus infection.

When pathogens infect laboratory animals, the immune system is activated regardless of the level of pathogenicity. Many micro-organisms have the potential to induce functional suppression or stimulation of the immune system. Sometimes, only T cells, B cells or specific subpopulations are influenced. Therefore, most virus infections and infections with bacteria or parasites are detrimental to immunological research and must be avoided.

Some micro-organisms have a specific effect on enzymatic or haematological parameters. Lactate dehydrogenase virus (LDV) can induce an up to 100-fold increase in the activity of lactate dehydrogenase (LDH) and other enzymes in the plasma. Numerous reports exist in the literature about

modulation of oncogenesis. Infectious agents may induce cancer, enhance chemical or viral carcinogenesis, or reduce the incidence of cancer. Some organisms even influence the growth rate of transplantable tumours.

Immunosuppressed animals are usually more sensitive to infections than are immunocompetent animals. Infections in immunodeficient animals frequently result in increased mortality due to a reduced or absent resistance to low pathogenic or even commensal micro-organisms.

It is important for various reasons that animals used for infection studies are free from adventitious infections. The infection in question might be influenced by an adventitious organism by means of immunomodulation and, therefore, result in increased or reduced resistance to experimental

Table 1. Mouse hepatitis virus (MHV): examples of interference with research

Effect of virus	Reference
Immunology	
Virus replication in macrophages, macrophage dysfunction	Boormann *et al.* (1982)
Dysfunction of T and B cells	de Souza *et al.* (1991)
Activation of NK cells, alteration of immune responsiveness	Schindler *et al.* (1982)
Immunosuppression or immunostimulation, depending on the time of infection	Virelizier *et al.* (1976)
Reduced levels of cytokines, γ-interferon and cytokines in spleen cells	de Souza *et al.* (1991)
Permanent decrease in skin graft rejection and T-cell-dependent antibody responses after recovery from infection	Cray *et al.* (1993)
Microbiology	
Reduced susceptibility to viral infections (Sendai, PVM)	Carrano *et al.* (1984)
Enhanced resistance to *Salmonella* infections	Fallon *et al.* (1991)
Confusion about the origin of Tettnang virus isolates	Smith *et al.* (1983)
Physiology	
Alteration of liver enzyme levels	Barthold (1986)
Altered protein synthesis	Lucchiari *et al.* (1992)
Changes in peripheral blood	Piazza *et al.* (1965)
Increased monocyte procoagulant activity	Levy *et al.* (1981)
Decrease in the incidence of diabetes in non-obese diabetic mice	Wilberz *et al.* (1991)
Oncology	
Abnormal tumour passage intervals or tumour invasion pattern	Manaker *et al.* (1961)
Rejection of human xenografts in the nude mouse	Kyriazis *et al.* (1979)
Contamination of transplantable tumours	Nicklas *et al.* (1993a)

NK, natural killer; PVM, pneumonia virus of mice.

infection. Micro-organisms resulting from a natural infection might contaminate viruses, bacteria or parasites that are passaged in laboratory animals. Spontaneous infections may lead to false conclusions. For example, the first isolations of Sendai virus were made from mice that had been inoculated with diagnostic materials from humans and swine. In subsequent years, evidence accumulated to show that an indigenous virus of mice had been isolated (National Research Council, 1991).

Some examples of virus interference with research are given in Table 1 for a mouse virus (MHV) and in Table 2 for a rat virus (Kilham rat virus (KRV)).

Table 2. Kilham rat virus (KRV): examples of interference with research

Effect of virus	Reference
Immunology	
Infection of T and B lymphocytes and suppression of various lymphocyte functions	McKisic *et al.* (1995)
Stimulation of autoreactive T lymphocytes specific for pancreatic antigens	Brown *et al.* (1993)
Altered susceptibility to autoimmune diabetes in rats	Guberski *et al.* (1991), Ellermann *et al.* (1996)
Altered cytotoxic lymphocyte activity	Darrigrand *et al.* (1984)
Depression of lymphocyte viability and various T-cell functions	Campbell *et al.* (1977a,b)
Stimulation of interferon production	Kilham *et al.* (1968)
Microbiology	
Supports secondary colonization with other micro-organisms	Carthew and Gannon (1981)
Influence on the prevalence of *Yersinia*-induced arthritis in rats	Gripenberg-Lerche and Toivanen (1993, 1994)
Persistent infection of cell lines	Wozniak and Hetrick (1969)
Physiology	
Inhibition of lipid formation in rat kidney cells *in vitro*	Schuster *et al.* (1991)
Increased leukocyte adhesion in the aortic epithelium	Gabaldon *et al.* (1992)
Congenital malformation	Margolis and Kilham (1975)
Death and resorption of fetuses	Kilham and Margolis (1966)
Oncology	
Suppression of leukaemia induction by Moloney virus	Bergs (1969)
Containment of leukaemias or leukaemia virus preparations	Spencer (1967)
Contamination of tumours	Campbell *et al.* (1977b)

Principles of Health Monitoring

The microbiological quality of laboratory animals is a direct result of colony management practices, and monitoring provides an after-the-fact assessment of the adequacy of those practices. Monitoring is, therefore, of greatest value in connection with maintenance of animals in isolation systems where vigorous microbiological control is applied.

Health monitoring procedures in animal populations differ from the procedures used in human medicine. Especially in populations of small laboratory animals, such as mice and rats, a single animal has only a limited value. Health monitoring of laboratory rodents aims at detecting health problems or defining the pathogen status in a population rather than in an individual. Therefore, systematic laboratory investigations (health surveillance programmes) are necessary to determine the colony status and, most importantly, to prevent influences on experiments. Disease diagnosis differs from monitoring in that abnormalities are the subject of testing. This testing is not scheduled, and tests are directed towards identifying those pathogens most likely to cause the lesion.

Routine monitoring programmes will primarily focus on infectious agents. Most infections are subclinical, but can nevertheless modify research results. Therefore, detection of the presence of infectious agents, whether or not they cause clinical disease, is necessary. Monitoring must include animals in the colony and all relevant vectors by which micro-organisms may be introduced into a colony. Therefore, it may be necessary, particularly in experimental units, that monitoring is not restricted to animals, and that other materials that pose a risk (e.g. biological materials) be monitored to prevent the introduction of agents into a facility.

The need for health surveillance programmes is generally accepted, but there is a great diversity of opinion about their design. Every institution requires an individual programme that has to be tailored to the conditions it is to serve. Most importantly, although the programme is dependent on research objectives, numerous additional factors must be considered, such as the physical conditions and layout of the animal house, husbandry methods and sources of animals. The type of programme further is influenced by the number as well as the quality of personnel, and by finances. It may even be necessary in a multipurpose unit to have a range of different programmes (e.g. one for isolator-housed and one for barrier-housed animals).

There is always a risk that infectious agents might be introduced, especially into experimental units. This risk has to be taken into consideration when the monitoring programme is designed. More frequent monitoring is reasonable if the risk of introducing unwanted organisms is high (e.g. if animals or biological materials are frequently introduced or if many personnel need access to the animals). Simulation experiments have shown that small and frequent samples are more suitable for detecting an infection than larger samples taken at less frequent intervals (Kunstyr, 1992).

Various designs of monitoring programmes have been published or presented on scientific meetings. General aspects of health surveillance

are provided by the Committee on Infectious Diseases of Mice and Rats (National Research Council, 1991). Recommendations exist about how monitoring of breeding colonies (Kraft *et al.*, 1994) or experimental colonies (Rehbinder *et al.*, 1996) should be conducted. An overview of the monitoring of experimental rodent colonies has been given by Nicklas (1996).

Animals

In general, the animals are the most crucial point in a monitoring programme. Their status has to be defined, and they are the most important source of infection. Proper sampling is therefore necessary in order to detect an infection in a given population as early as possible. Animals coming from outside have to be checked to assess or exclude the risk of introducing unwanted organisms, and animals already within the unit are monitored to define their status and to obtain information on the presence or absence of infectious agents in the colony. It is obvious that a sufficient number of animals has to be monitored. Based on a recommendation by the ILAR Committee on Long-Term Holding of Laboratory Rodents (1976), it has become common practice to monitor at least eight randomly sampled animals, which is (theoretically) sufficient to detect an infection with a 95% probability if at least 30% of a population is infected. Monitoring animals of different ages is useful, because younger animals often have a greater parasite or bacterial burden, whereas older animals ($\geq$3 months) are more suitable for detecting viral infections.

Sentinels/'control' animals

Random sampling for monitoring is not a serious problem in breeding colonies, but it is usually impossible in experimental units or not reasonable in the case of immunodeficient animals. Immunodeficient animals may not be able to produce sufficient amounts of antibodies, and so their status can be evaluated only by the use of sentinels. It is therefore advisable to have sentinel animals in each experimental unit in order to evaluate the status of a population. Such animals should be kept in such a way that they receive maximum exposure to potential infections. If sentinels are not bred within the colony that is being monitored, they must be obtained from a breeding colony of known microbiological status, i.e. they must be negative for all rodent pathogens. The sentinel animals must be housed for a sufficiently long time in the population that is to be monitored in order to develop detectable antibody titres (for serology) or parasitic stages. It is common to house sentinels in a population for at least 4–6 weeks prior to testing, longer periods are even better. In most cases, outbred animals are used as sentinels, because they are cheaper and more resistant to clinical disease than are inbred animals. Inbred animals may in specific cases (e.g. for virus isolation) be more valuable as sentinels, because they may be more sensitive to an agent and thus more likely to develop clinical disease. In other cases, their extreme

or even complete resistance to specific agents may be a reason to use specific strains with known characteristics. For example, C57BL/6 or DBA/2 mice are sensitive to clinical infections with MHV, whereas A/J mice are resistant to this virus. On the other hand, C57BL/6 mice are resistant to ectromelia virus (Bhatt and Jacoby, 1987). This virus causes high mortality with typical skin lesions in C3H mice, and high mortality but minimal skin lesions in CBA and DBA/2 mice. Use of immunodeficient animals, such as thymus-aplastic nude mice, as sentinels may increase the sensitivity if specific bacterial pathogens such as *Pasteurella pneumotropica*, parasites (e.g. *Spironucleus muris*) or viruses are to be detected in a population. In the past, injection of cortisone to suppress the immune system was recommended. Cortisone results in overgrowth and thus makes it easier to detect bacterial pathogens directly. However, cortisone tests have lost importance as the direct demonstration of microorganisms can now be performed more easily by means of molecular methods such as the polymerase chain reaction (PCR).

A multitude of physiological characteristics can be influenced by introducing a transgene into the genome or by gene targeting. Changes of the immune status frequently arise, resulting in immune defects or immunosuppression. As a consequence, there may not only be altered sensitivity to pathogenic agents, but also suppression or lack of antibody response. When monitoring an immunodeficient colony, to avoid false-negative results in serological tests animals whose immune responsiveness is well known (e.g. old vasectomized males, retired breeder females) should be used as sentinels in order to obtain reliable serological results. It is advisable for classical barrier systems to have sentinel animals in each animal room. The animals should be housed in various locations on the bottom shelves, without filter tops. Each time the cages are changed, soiled bedding from different cages should be transferred to sentinel cages.

During the last decade additional housing systems such as microisolators, individually ventilated cages and filter cabinets (see pages 134–135) have emerged. These offer the advantage of separating small populations from each other and are frequently used for housing immunodeficient, immunosuppressed or infected animals, because they very efficiently prevent transmission of infectious agents. Each isolator or microisolator cage must therefore be considered as a self-contained microbiological entity. Health monitoring under such housing conditions as well as monitoring isolator-housed animals can only be conducted by the use of sentinel animals. Due to limited space, less than the recommended number of animals are available in many cases, which is acceptable if sentinels are properly housed. In the case of isolators, a realistic number of sentinel animals is housed in one or several cages (depending on the isolator size) on soiled bedding taken from as many cages as possible. In most cases, only 3–5 animals per isolator will be available for monitoring.

If animals are housed in microisolators or in individually ventilated cages, sentinels must be housed in filter-top units like other animals. When cages are changed in changing cabinets, soiled bedding from several cages is transferred into a separate cage which is used to house

sentinels. Weekly changes of donor cages will give a representative insight into the microbiological status of the whole population.

Frequency of monitoring

The frequency of monitoring will depend on various factors, but mainly on the importance of a pathogen to the use of the population and on the level of risk of infection for a population. Naturally, economic considerations are important as well. Both of the recommendations of the Federation of European Laboratory Animal Science Associations (FELASA) (Kraft *et al.*, 1994; Rehbinder *et al.*, 1996) state that monitoring should be conducted quarterly. Most commercial breeders of laboratory rodents monitor more frequently (every 4–6 weeks). In most multipurpose units housing immunodeficient or infected animals, more frequent monitoring is preferable as this will result in earlier detection of an infection. As a general rule, it is advisable to monitor a small number (e.g. 3–5) of animals from each unit every 4–6 weeks instead of 10 animals every 3 months. Under practical conditions, not every animal may be monitored for all micro-organisms. Depending on the factors already mentioned, the frequency of testing may be different for different agents. Monitoring for more frequently occurring organisms or for zoonotic or otherwise important agents will be performed more frequently (monthly), whereas testing for unusual organisms like K-virus or polyoma virus can be done less frequently (e.g. biannually or annually). Results obtained from monitoring of sentinels are valid for all animals of the same species within a population, irrespective of the experiment or animal strain. Independent from animals which are scheduled for monitoring, all animals with clinical disease should be submitted for direct examination for micro-organisms (bacteria, parasites, viruses) and for histopathology.

Biological materials

In addition to animals, other materials may carry unwanted micro-organisms and may be important sources of infection. Immunodeficient nude mice are often used for tumour transplantation studies and are at risk of infections transmitted via the transplanted tissue. In many cases, organisms have been introduced into animal populations by contaminated tumours or leukaemias (Collins and Parker, 1972; Nicklas *et al.*, 1993a). Monoclonal antibodies (Nicklas *et al.*, 1988) and virus suspensions (Smith *et al.*, 1983) used for infection studies might also be contaminated, and these must be monitored before use in animals.

Agents

A decision has to be made in each facility about which organisms are acceptable or unacceptable. Lists of infectious agents to be monitored in routine programmes have been published by various organizations (Kunstyr, 1988a; National Research Council, 1991; Kraft *et al.*, 1994; Waggie *et al.*, 1994) and can be used for guidance. Monitoring for all the

agents mentioned (mycoplasmas, bacteria, bartonellas, fungi, spirochaetes, protozoans, helminths, arthropods) on a routine basis is neither realistic nor necessary. The most important micro-organisms are those that are indigenous and pose a threat to the research or to the health of the animals and humans and, in addition, those which can be eliminated. Therefore, oncogenic retroviruses are excluded as they integrate into the mammalian genome, and thus cannot be eradicated by presently available methods. Other micro-organisms may be less important as they are unlikely to occur in good quality rodents due to repeated rederivation procedures (e.g. *Brucella, Erysipelothrix*). Most cestodes are unlikely to be found, since they require an intermediate host. In the case of immunocompromised animals or in infection experiments, however, monitoring for a comprehensive list of micro-organisms (some examples are given in Box 1) is reasonable. Various micro-organisms that usually do not cause clinical signs in immunocompetent animals (e.g. *Staph. aureus, Pseud. aeruginosa, Pneum. carinii*) may cause serious problems in immunodeficient animals. It is

Box 1 Examples of bacterial and fungal pathogens and parasites that should not be detectable in barrier-housed colonies of mice and rats

Bacteria
Actinobacillus muris
Actinobacillus sp.
Bordetella bronchiseptica
CAR bacillus
Citrobacter rodentium
Clostridium piliforme
Corynebacterium kutscheri
Erysipelothrix rhusiopathiae
Haemophilus sp.
Haemophilus influenzaemurium
Helicobacter sp.
Klebsiella pneumoniae
Klebsiella oxytoca
Listeria monocytogenes/ivanovii
Pasteurella multocida
Pasteurella pneumotropica
Other Pasteurellaceae
Pseudomonas aeruginosa
Salmonella sp.
Staphylococcus aureus
Streptobacillus moniliformis
Streptococcus pneumoniae
β-Haemolytic Streptococci
Yersinia pseudotuberculosis

Mycoplasmas
Mycoplasma pulmonis
Mycoplasma arthritidis
Mycoplasma neurolyticum

Fungi
Trichophyton sp.
Microsporum sp.
Yeasts

Parasites (all parasites)
Aspiculuris tetraptera
Syphacia obvelata
Syphacia muris
Trichosomoides crassicauda
Hymenolepis sp.
Spironucleus muris
Coccidia
Giardia sp.
Trichomonads
Amoebae
Demodex sp.
Myobia musculi
Myocoptes musculinus
Notoedres sp.
Polyplax spinulosa
Radfordia affinis
Radfordia ensifera

therefore necessary that immunodeficient animals are monitored not only for strong or weakly pathogenic organisms, but also for opportunistic pathogens or commensals. Micro-organisms with a low pathogenic potential can cause clinical signs of disease if animals are infected with several agents (e.g. KRV and *Past. pneumotropica* (Carthew and Gannon, 1981)). In other cases, different micro-organisms of low clinical importance may interact and have a severe impact on research results such as oncogenic viral expression (Riley, 1966).

Each institution should prepare a list of those organisms that are not acceptable in the colony or in parts of it. The list is easiest to establish for viruses (for an example, see Table 3). A large amount of information is available on their pathogenic potential and on their ability to compromise the object of research. Monitoring for viruses can be done selectively by serological methods. Only a few exceptions exist, e.g. parvoviruses that cross-react in indirect immunofluorescence or enzyme-linked

Table 3. Serologic tests for the detection of infectious agents in mice and rats

Infectious agent	Recommended methods	Species
Viruses		
Mouse hepatitis virus (MHV)	ELISA, IIF	Mouse
Rat corona viruses (RCV/SDAV)	ELISA, IIF	Rat
Kilham rat virus (KRV)	HI, ELISA, IIF	Rat
Toolan's H-1 virus	HI, ELISA, IIF	Rat
Minute virus of mice (MVM)	HI, ELISA, IIF	Mouse
Pneumonia virus of mice (PVM)	ELISA, IIF, HI	Mouse, rat
Reo virus type 3	ELISA, IIF	Mouse, rat
Sendai virus	ELISA, IIF, HI	Mouse, rat
Mouse encephalomyelitis virus (GD VII)	ELISA, IIF, HI	Mouse, rat
Mouse adenovirus (FL, K87)	ELISA, IIF	Mouse, rat
K-virus	HI	Mouse
Polyoma virus	ELISA, IIF, HI	Mouse
Lymphocytic choriomeningitis virus (LCMV)	ELISA, IIF	Mouse
Ectromelia virus	ELISA, IIF	Mouse
Hantaviruses	ELISA, IIF	Rat
Mouse rotavirus (EDIM)	ELISA, IIF	Mouse
Lactic dehydrogenase elevating virus (LDV)	PCR, enzyme test	Mouse
Bacteria		
Mycoplasma pulmonis	ELISA, IIF, culture	Mouse, rat
Mycoplasma arthritidis	ELISA, IIF, culture	Rat
Clostridium piliforme	IIF	Mouse, rat
CAR bacillus	ELISA	Rat

ELISA, enzyme-linked immunosorbent assay; HI, haemagglutination inhibition assay; IIF, indirect immunofluorescence assay; PCR, polymerase chain reaction.

immunosorbent assay (ELISA) tests (Jacoby *et al.*, 1996) and sometimes cannot be identified unequivocally. For some viruses (e.g. K virus, polyoma virus) the only question is whether or not monitoring is necessary, because they have been eradicated from the vast majority of rodent colonies many years ago. Only few new rodent viruses have been detected during the last few years, e.g. mouse parvovirus (MPV) and rat parvovirus (RPV) (Jacoby *et al.*, 1996), and it has to be expected that new rodent viruses will be isolated, although only occasionally.

Less is known about the ability of most parasites to influence research results. They are considered to be a hygiene problem and are therefore eradicated from rodent colonies. Some protozoans, such as trichomonads, are occasionally detectable in pathogen-free animals from commercial breeders. They are considered to be apathogenic, and nothing is known about their influence on the physiology of animals. They are, however, likely to be species specific, and thus might be an indicator of a leak in the system or of the existence of direct or indirect contact with wild rodents. The most complex problems exist for bacteria. In contrast to viruses their importance in laboratory animals is usually estimated on the basis of their ability to induce pathological changes or clinical disease, since almost nothing is known about most rodent bacterial species with regard to their potential to cause other effects on their hosts and on experiments. Insufficient information exists on the taxonomy and proper identification for various rodent-specific bacterial species such as *Past. pneumotropica* or other members of the Pasteurellaceae (e.g. *Haemophilus influenzaemurium, Actinobacillus muris*). Lack of detailed information on the characteristics of these organisms together with the presently unclear taxonomic situation often leads to misidentification, and the lack of knowledge about species specificity impedes their elimination. The FELASA working group on animal health (Rehbinder *et al.*, 1996) therefore decided to recommend that rodents should be monitored for all Pasteurellaceae. There is, however, evidence, that some growth-factor-dependent Pasteurellaceae found in rodents are closely related to *Haemophilus parainfluenzae* and might therefore be transmitted by humans (Nicklas *et al.*, 1993b). It is unclear if these bacteria can be eradicated permanently from barrier units, because exposure of barrier-produced animals to humans represents a permanent risk for reinfection. The same is true for several members of the Enterobacteriaceae (*E. coli, Klebsiella, Proteus), Staph. aureus* and *Pseud. aeruginosa*, for which humans are the reservoir. Another problem arises from the fact that many bacteria are presently being reclassified, resulting in changes in their names. For example, the mouse-specific organism known as *'Citrobacter freundii* 4280' has recently been reclassified as *Citrobacter rodentium* (Schauer *et al.*, 1995). Whole genera have been renamed, and additional bacterial species have been detected, e.g. *Helicobacter hepaticus, Heli. muridarum* and *Heli. bilis* (Lee *et al.*, 1992; Fox *et al.*, 1994, 1995). Some of these fastidious organisms are not detected or not properly identified by all monitoring laboratories. Adding such known pathogens to a list for which animals should be monitored may be unrealistic as long as proper methods for their detection and identification are not readily available in a monitoring laboratory.

A list of pathogens should contain all indigenous micro-organisms for which rodents are the infectious reservoirs and other micro-organisms that might be of importance for the research conducted with such animals. The list of these additional organisms may be long in the case of immunodeficient animals. The whole spectrum of micro-organisms as a concept is not a permanent list for all time, it rather represents a moving boundary in which old pathogens are eradicated and new pathogens are added. In practice, such lists of agents do not differ much between different facilities or commercial breeders. Monitoring for micro-organisms is usually done by commercial laboratories, and is thus determined by their capabilities (some of the larger research institutes have dedicated diagnostic laboratories). It is important that all investigations should be performed in laboratories with sufficient expertise in microbiology or pathology of the relevant species. Serological tests also require technical competence to ensure sufficient standardization of tests (including controls) and accurate interpretation of results.

Testing of animals usually starts with necropsy and blood sampling for serology, followed by microscopic examination for parasites and sampling of organs for bacteriology, pathology and, in rare cases, virological examinations. For financial reasons, bacterial culture is often restricted to very few organs. Monitoring more organs would, however, increase the probability of detecting bacterial pathogens in an animal. Bacterial cultures should be done for the respiratory tract (nasal cavity, trachea, lungs), intestinal tract (small and large intestine) and urogenital tract (vagina, prepuce, uterus, kidney). In the case of pathological changes, additional organs (liver, spleen, mammary gland, lymph nodes, conjunctiva, etc.) should be cultured.

Serology is easy and cheap to perform, and serum samples can be mailed easily. Whole-body examinations including bacteriology and parasitology are more expensive, and live animals must be shipped to the monitoring laboratory. Therefore, many laboratories monitor only serologically. Meanwhile, serological methods exist to detect some bacterial infections, but these are not generally accepted, and only a few laboratories apply these methods. At present, the method of choice for the detection of most bacterial pathogens is bacterial culture, and thus should be part of every monitoring programme.

Sources of Infection

Keeping rodents free of pathogens in research facilities is a much more complex problem than in breeding colonies. Animals and various experimental materials need to be introduced into experimental facilities. In addition, more personnel must have access to animals due to the requirements of the experiments. This results in a higher risk of introducing pathogens.

Effective measures must be taken to standardize laboratory animals microbiologically as far as possible. Therefore, the design of modern laboratory animal buildings is based mainly on microbiological concepts aimed at the prevention of infections. These measures are responsible for

a high percentage of the expense arising from planning and constructing an animal house. Furthermore, high running costs are taken into account for energy, hygienic precautions, and personnel to avoid infections during operation.

In addition to constructive measures, an appropriate management system is necessary for the prevention of infections, as well as for their detection and control. It is a major task for the management of an animal facility to understand how micro-organisms might be introduced or spread under the specific conditions given. Management of all animal facilities in an institution is best centralized. This warrants that all information dealing with the purchase of animals, use of experimental materials and equipment, as well as the performance of animal experiments flows through one office. This reduces the opportunity for failures of communication. Centralized management can best establish comprehensive monitoring programmes to evaluate important risk factors such as animals and biological materials before they are introduced into a facility. Contamination of animals can happen in two ways. One has to distinguish between the introduction of micro-organisms coming from outside and the transmission of micro-organisms within a colony. Both can be influenced by the management and the housing system.

Animals

The greatest risk of contamination of any animal arises from another animal of the same species. Most facilities are multipurpose, and must therefore house a variety of strains coming from various breeding units. In addition, many specific strains or transgenic animals are available only from research institutes. Still, animals are the most important risk factor, even if their quality has constantly improved during the last decades.

As a general rule, all animals coming from sources of unknown microbiological status should be regarded as infected unless their status has been defined. This is especially important when transgenic animals are introduced from other experimental colonies. These animals must be housed separately from others. The risk of introducing pathogens via animals from external sources is lower when animals are available from very few sources of well-known microbiological status and if these animals have been protected from contamination during shipment. In many cases direct transfer of such animals without quarantine into an experimental unit will be necessary; however, spot checks should be performed from time to time to redefine the status upon arrival. In many cases it is acceptable to introduce animals from microbiologically well-known (external) colonies into experimental units, but never into a breeding unit, especially if many different strains and/or transgenic lines are co-maintained. In the latter case new breeders should only be introduced via embryo transfer or hysterectomy (see pages 162–163). Outbred mice or F1 hybrids are generally used as surrogate and foster dams and can easily be bred in the transgenic unit, as is the case for the sterile males required to induce pseudocyesis in the surrogate dams.

It must be emphasized that a specific risk of transmitting micro-organisms may arise from immunodeficient animals. Many virus infections (MHV, RCV/SDA, Sendai, PVM) are limited in immunocompetent animals, and the virus may be eliminated completely. Immunodeficient animals may, however, shed infectious virus for longer periods of time, or may be infected persistently.

Like animals of unknown status, animals known to be infected must always be housed in isolation. This can best be done in isolators or, if proper handling is guaranteed, in microisolator cages or in individually ventilated cages.

Biological materials

Biological materials represent a high risk if they originate from or have been propagated in animals. In particular, tumours, viruses or parasites that are serially passaged in animals often pick up pathogens, and therefore a high percentage of these are contaminated. Many murine viruses (e.g. MVM, K virus, mouse encephalomyelitis virus and mouse adenovirus) were first isolated from contaminated virus pools or (e.g. polyoma virus, Kilham rat virus (KRV), Toolan's H-1 virus) from contaminated tumours. Such materials can be stored frozen without loss of infectivity, and may be hazardous to humans or laboratory animals even after decades. The problem of viral contamination in biological materials became obvious in the studies done by Collins and Parker (1972). They monitored 475 murine leukaemias and tumours and found viral contamination in 69% of the samples. The same percentage of contaminated mouse tumour samples was found by Nicklas *et al.* (1993a) after animal passages. Many organisms disappear under *in vitro* conditions, so that the contamination rate after these passages is lower. Among the contaminants, lymphocytic choriomeningitis virus (LCMV) (Bhatt *et al.*, 1986b) and hantaviruses (Yamanishi *et al.*, 1983) have repeatedly been found, and outbreaks in humans associated with infected animals or with contaminated tumour material have been reported (Kawamata *et al.*, 1987).

Pathogenic micro-organisms can also be transmitted by other contaminated materials of animal origin, such as monoclonal antibodies (Nicklas *et al.*, 1988) and viruses (Smith *et al.*, 1983). Contamination of biological materials is not restricted to viruses. *Myc. pulmonis* and other bacterial pathogens such as *Past. pneumotropica* have been found in tumours (Nicklas, 1993). Additional pathogens (*Eperythrozoon* sp., *Haemobartonella* sp., *Encephalitozoon* sp.) can contaminate biological materials after animal-to-animal passage (National Research Council, 1991) and thus may be transmitted to recipient animals.

Humans

Humans can act as mechanical or biological carriers of micro-organisms. Humans are unlikely to be an appropriate host where murine pathogens

can reside and replicate. However, the importance of humans as mechanical vectors should not be underestimated, and several human pathogens can cause infections in rodents, at least in immunodeficient animals. It has to be assumed that each micro-organism that is present in humans who have access to a barrier unit might sooner or later colonize the animals. Transmission certainly cannot be avoided in barrier-maintained colonies, even by wearing gloves and surgical masks and taking other precautions. It may only be avoided by establishing strict barriers as provided by isolator maintenance. Immunodeficient animals, at least animals used for breeding or long-term experiments, which are known to have an increased sensitivity to infection with bacteria of human origin (*Staph. aureus, Kleb. pneumoniae, Esch. coli*, etc.) should, therefore, be housed in isolators or microisolators (individually ventilated cages).

Little published information is available on the role of humans as mechanical vectors. There is no doubt that micro-organisms can be transmitted by handling (La Regina *et al.*, 1992). Micro-organisms can even be transported from pets to laboratory animals by human vectors (Tietjen, 1992). Such examples emphasize the need for proper hygienic measures and the importance of positive motivation of staff. It is an important task of the management of an animal facility to ensure that personnel coming into contact with animals have no contact with animals of lower microbiological quality.

Vermin

Vermin are another potential source of infections. Flying insects do not present a serious problem because they can easily be removed from the incoming air by means of filters or insect-electrocuting devices. Crawling insects such as cockroaches are more difficult to control, and cannot be excluded with certainty. The most serious problem arises from wild rodents, which are frequently carriers of infections. Wild, as well as escaped, rodents are attracted by animal diets, bedding and waste. Modern animal houses usually have devices that normally prevent entry of vermin.

Possible routes of infection of laboratory animals have been discussed in more detail by Nicklas (1993).

Present Status of Laboratory Animals

Since serological testing has been possible, many laboratories have evaluated the murine viral status of rodent colonies. Managers of animal facilities had to learn techniques to prevent, control and eradicate infection and means of adapting the facilities for their own purposes. As a consequence, the diversity of viruses and the frequency with which they are detected has declined markedly. Virus infections have now been almost entirely eradicated from most commercial breeding colonies. This

gave animal care unit administrators and researchers the opportunity to procure and maintain virus-free stocks, and researchers to use better standardized animals for research. However, this progress of eradication has not occurred without periodic shut-downs at breeders' and users' facilities.

Reports on the prevalence of virus infections in rodents throughout the world have been published frequently. An overview given by the National Research Council in 1991 demonstrates that the majority of colonies were at that time infected with 3–4 viruses. It has to be expected that more recent statistics would reveal that the prevalence of murine viruses has declined further. However, most facilities house at least small numbers of infected animals or animals of unknown status. Many small or decentralized facilities do not monitor at all. Today, murine parvoviruses and MHV are the most prevalent agents in rodents. Especially for parvoviruses the situation is unclear, because recently described parvoviruses have not yet been sufficiently characterized, and only limited knowledge exists about their prevalence. Various viruses are still prevalent at a low level. These can emerge unexpectedly, as occurred a few years ago when a sudden outbreak of ectromelia was observed in the USA (Dick *et al.*, 1996). This virus had not been detected in the USA for many years.

The situation is very similar for bacterial pathogens and parasites. Most of these were eradicated when the principles of gnotobiology were introduced into laboratory animal science. A few parasites (pinworms, mites, protozoans) are still endemic in various rodent colonies, but most of the primary bacterial pathogens (Salmonellae, *Corynebacterium kutscheri, Leptospira, Streptobacillus moniliformis*) are no longer detected in well-run facilities, although they may re-emerge as shown recently (Wullenweber *et al.*, 1990; Koopman *et al.*, 1991). *Clostridium piliforme*, which is the causative agent of Tyzzer's disease, and *Myc. pulmonis*, are detected more frequently. Most experimental colonies and some commercial breeders' colonies are positive for Pasteurellaceae like *Past. pneumotropica* and *Actinobacillus muris*. The real prevalence of organisms belonging to this family is not definitely known, due to difficulties in identification. The situation is also unclear for *Helicobacter* species, because these cannot be detected in all monitoring laboratories. It has to be expected that these, too, are widespread in laboratory rodents. Such organisms have in the past been spread by animals that had become infected long before the organisms had been detected. It is, therefore, extremely important that germ-free or gnotobiotic animals, rather than SPF animals, are used for hygienic rederivation in order to avoid this problem in the future.

A number of additional disease agents such as group B and G streptococci, *Staph. aureus, Haem. parainfluenzae, Corynebacteria* spp. inducing scaly skin disease, and others have been found in so-called pathogen-free rodents during the last few years. Rodents seem not to be the primary hosts for these organisms, and they are likely to be transmitted by humans. These infections have been named 'post-indigenous diseases' (Weisbroth, 1996).

The presence of infectious agents, even if they are of low pathogenicity, may become a problem if animals from different sources are housed together. This occurs often, as transgenic animals are frequently exchanged between scientists from an almost unlimited number of sources. This is associated with a high risk of introducing different pathogens and thus of causing multiple infections. At present, infections that were common decades ago are re-emerging.

◆◆◆◆◆ IMMUNOCOMPROMISED ANIMALS

Nature has produced quite a variety of mutations affecting the immune system of mice and rats. Some of the deficiencies have been shown to be complex, involving several genes rather than being determined by a single point mutation (e.g. *Prkdc*^{scid}, *Hfh11*^{nu}). Despite phenotypic similarities the genetic basis of various mutations must not be the same. The genetic factors coding for similar phenotypes may act at different developmental stages or differentiation steps (e.g. *Prkdc*^{scid}, *Rag1*/*Rag2*). However, it should be kept in mind that the phenotypic appearance of a mutation might be strikingly altered by the genetic background of the mutant-bearing strain, as has been reported for a large body of examples. While *db*/*db* mice on a C57BL/Ks background (strain of origin) develop an early-onset diabetes that resembles in some respects human non-insulin-dependent diabetes mellitus (type II), the C57BL/6J background has been shown to be diabetes resistant (Coleman, 1978; Leiter *et al.*, 1979). Another example is mice that lack the epidermal growth factor receptor (EGFR). On the genetic background of 129/Sv, mutant fetuses are retarded and die at midgestation; whereas on a mixed background like 129/Sv × C57BL/6 or 129/Sv × C57BL/6 × MF1, fetuses survive until birth and to postnatal day 20, respectively (Sibilia and Wagner, 1995). Mice carrying a null-mutation of the interleukin-2 gene (*IL2*^{tm1Hor}) on the original 129/Ola × C57BL/6 background (129,B6) develop normally during the early postnatal period until about weaning. Thereafter, immunodeficiency becomes evident and the mice die within the next 4 weeks or develop an inflammatory bowel disease (Sadlack *et al.*, 1993). If this knock-out mutation is transferred onto a BALB/c background the lifespan is considerably shortened, with none of the mutants surviving the third week: in C3H/HeJCrl-*IL2*^{tm1Hor} death occurs by 7 weeks and in C57BL/6J-*IL2*^{tm1Hor} by 12–24 weeks of age (Mähler *et al.*, 1996). Such effects of the host genome on the expression pattern of genetically defined single-locus mutations must always be considered, not only when setting up experiments, but also when establishing a new mutation by transferring it onto a given genetic background.

The relative ease of breeding small laboratory rodents also allows for the combination of various mutations and thus of providing experimental animals that are suitable for specific studies. It is not possible to summarize here all the available information and key references on the genetics, pathophysiology, husbandry and reproduction of the abundant natural and induced hereditary immunodeficiencies in rodents – the latter

are growing exponentially in number due to the establishment of new molecular biology techniques. Thus only a rough outline is provided.

Investigators must also be aware that certain environmental factors, both infectious and non-infectious, can lead to transient or persistent suppression or stimulation of the immune system. Such factors (e.g. chlorinated drinking water, tetracycline, infections with MHV or lactate dehydrogenase virus) may complicate research results, regardless of whether the animals are immunodeficient or of wild type (+/+), and should be avoided.

Variants Produced by Nature

Naturally occurring immunodeficient mouse strains express a variety of genetic defects in myeloid and/or lymphoid cell development. These strains have served as, and still are valuable models for, studying immune cell differentiation, mechanisms of transplant rejection, etc. Some of the most commonly used mutants are nude (*Hfh11*^{nu}), severe combined immunodeficiency (*Prkdc*^{scid}), beige (*Lyst*^{bg}), and X-linked immunodeficiency (*Btk*^{xid}). Information about the different variants produced by nature can be found in an ILAR guide (ILAR Committee on Immunologically Compromised Rodents, 1989), Lyon *et al.* (1996) and Hedrich (1990), or by searching for defined mutations in databases such as Mouse Genome Database (http://www.informatics.jax.org) and RATMAP (http://ratmap.gen.gu.se). Tables 4 and 5 give a selection of immunodeficient mutants in laboratory rodents that are often used. Apart from their immunodeficient status (i.e. their inability to eliminate or neutralize foreign substances), some mutants also inherit a failure to discriminate between self and non-self.

In addition to the action of defined genes on the immune function there are several inbred strains or F1 hybrids harbouring genes that confer susceptibility or resistance to infectious or other immune-system-related diseases. As an example, C57BL/6 and related strains succumb to infection with *Streptobacillus moniliformis*, AKR, BALB/c, DBA/2 and other mice survive, while BALB/c mice never show any sign of disturbance, nor even produce antibodies against this organism (Wullenweber *et al.*, 1990).

Variants Produced by Genetic Manipulation

The advent of transgenic rodent technology by transferring and overexpressing foreign genes under the control of specific vectors as well as directed mutagenesis by silencing specific genes has opened up new avenues to study innumerable factors that affect the immune system.

One may search for these either by consulting literature databases, the Mouse Genome Database (MGD; http://www.jax.org/resources; check 'Induced Mutant Resources'), or the Transgenic Animal Database (TBASE; http://www.gdb.org/dan/tbase/tbase.html). Again, as indicated above, identical phenotypes do not necessarily indicate identity of

Table 4. Hereditary immunodeficiencies in mice*

Mutation			Dysfunctions other than immunodeficiency		Care	
Locus	Name	Chromosome	Autoimmunity	None – immune	Special breeding	Special husbandry
bg (*Lyst*^{bg})	Beige	13	–	–	–	–
Dh	Dominant hemimelia	1	–	+	+	–
gl	Grey–lethal	10	–	+	+	–
gld (*FasL*^{gld})	Generalized lymphoproliferative disease	1	+	–	–	–
Hc	C'5 deficiency	2	–	–	–	–
hr	Hairless	14	–	+	+	–
lpr (*Fas*^{lpr})	Lymphoproliferation	19	+	–	+	–
lps	Defective lipopolysaccharide response	4	–	–	–	–
me (*Heph*^{me})	Motheaten	6	+	+	+	–
nu (*Hfh11*^{nu})	Nude	11	+	+	+	+
oc	Osteosclerosis	19	–	+	+	+
op (*Csfm*^{op})	Osteopetrosis	3	–	+	+	+
scid (*Prkdc*^{scid})	Severe combined immunodeficiency	16	–	–	–	+
W (*Kit*^{w})	Dominant spotting	5	–	+	+	–
xid (*Btk*^{xid})	X-linked immunodeficiency	X	–	–	–	–
Yaa	Y-linked immunodeficiency	Y	+	+	–	–

* For references, see Lyon *et al.* (1996) and http:www.informatics.jax.org./mgd.html.

If special breeding regimes are advisable this is indicated by + and indicates, for example, mating of (tested) heterozygotes, continued back-crossing, or even ovarian transplants to histocompatible hosts. Special husbandry (+) means either the need for an SPF environment, for special diets or other environmental conditions; (–) does not imply that, for example, SPF conditions are not required.

Table 5. Hereditary immunodeficiencies in rats, guinea-pigs and Syrian hamsters*

Mutation			Dysfunctions other than immunodeficiency		Care		Ref.
Locus	Name	Chromosome	Autoimmunity	None – immune	Special breeding	Special husbandry	
Rat							
an-2 (b)	Anaemia 1	5	–	+	+	–	Hedrich (1990)
C4	C4 deficiency	20	–	–	–	–	Hedrich (1990)
C6	C6 deficiency	?	–	–	–	–	Hedrich (1990)
ia	Incisor absent	5	–	+	+	+	Hedrich (1990)
l (Lyp)	Lymphopenia	4	–	(+)	–	(+)	Hedrich (1990)
mk	Masked	?	–	+	(+)	+	Hedrich (1990)
op	Osteopetrosis	?	–	+	+	+	Hedrich (1990)
rnu ($Hfh11^{rnu}$; whn^{rnu})	Nude	10	–	+	+	+	Hedrich (1990)
tl	Toothless	?	–	+	+	+	Hedrich (1990)
Guinea–pig							
C2	C2 deficiency	Mhc-linked	(+)	–	–	–	Bitter-Suermann *et al.* (1981) Böttger *et al.* (1986a)
C3	C3 deficiency	?	–	–	–	+	Böttger *et al.* (1985) Böttger *et al.* (1986b)
C4	C4 deficiency	?	(+)	–	–	–	Böttger *et al.* (1986a) Peltier (1982)
Syrian hamster							
C6	C6 deficiency	?	–	+	–	–	Yang *et al.* (1974)
nu	Nude	?	–	–	+	+	Loridon-Rosa *et al.* (1988)

* See footnote to Table 4.

the genes. *Prkdc*^{scid}, *Rag1*$^{-/-}$ and *Rag2*$^{-/-}$ deficient mice, which in many respects are phenotypically alike, have been shown to be different in terms of the genetic factors that control the expression of these immunodeficiencies. On the other hand, silencing of exon 3 of the *whn* gene has produced exactly the same phenotype as in *Hfh11*^{nu} mice, providing evidence that the fork-head transcription factor is responsible for both the nude and the athymic phenotype (Nehls *et al.*, 1996). Table 6 lists a few of the innumerable immunocompromised mutants that have been created in the recent years. PCR protocols that can be used to distinguish between mice carrying an induced mutation (maintained at the Jackson Laboratory) and normal wild-type mice are available on the World Wide Web (http://www.jax.org/resources/documents/imr/protocols/index.html) or through an e-mail inquiry to micetech@aretha.jax.org.

It should be noted that transgenic animals can only be maintained at or be supplied to premises that comply with the national requirements of the respective host country for the use of genetically modified animals.

◆◆◆◆◆ MANAGEMENT OF COLONIES

Housing Systems

The original descriptions of housing systems for small rodents have not lost their principal validity (see e.g. Spiegel, 1976; Otis and Foster, 1983; ILAR Committee on Immunologically Compromised Rodents, 1989), although many refinements have been introduced. In principle, the following different hygienic levels are distinguished: conventional, with no or low precautions; specified pathogen-free (SPF); gnotobiotic and germ-free; presumed infected 'quarantine', and infected. The different hygiene levels require different levels of precaution and presume adequate housing systems, which are used in the opposite safety version for quarantine and infected animals. The housing systems described below have different prerequisites in terms of the construction of the building and equipment. Their running is more or less labour and cost-intensive. The decision about the scientific requirements to be met must be made with respect to international standards.

Conventional

According to 'Good laboratory animal practice' (National Research Council, 1996), climatization of rooms, light cycle, standardized food, special bedding, adequate equipment, prevention of wild mice from entering the animal rooms, food and bedding stores, acceptable animal density and careful handling of the animals are inevitable prerequisites for running a conventional colony. The conventional system should be improved by basic hygienic precautions (e.g. overshoes, overalls, and hand washing). The colony should be monitored regularly in order to detect infections, which may influence the experimental results or the

Table 6. Some transgenic and gene targeted mice*

Mutation		Dysfunctions other than immunodeficiency		Care		
Locus	Chromosome	Autoimmunity	None – immune	Special breeding	Special husbandry	Ref.
β2m	2	–	–	–	–	Koller *et al.* (1990)
Btk		–	–	–	–	Khan *et al.* (1995)
Cd4	6	–	–	–	–	Locksley *et al.* (1993)
Hfh11^{nu} (*whn*)	11	+	+	+	+	Nehls *et al.* (1996)
Tcrα	14	–	+	–	+	Mombaerts *et al.* (1992a)
Tcrβ	6	–	+	–	+	Mombaerts *et al.* (1992a)
Icam1	9	–	–	–	(+)	King *et al.* (1995); Sligh *et al.* (1993) Xu *et al.* (1994)
IL2	3	–	+	–	(+)	Mähler *et al.* (1996); Sadlack *et al.* (1993)
IL4	11	–	+	–	(+)	Kühn *et al.* (1991); Metwali *et al.* (1996) Noben-Trauth *et al.* (1996)
IL6	5	–	–	–	(+)	Kopf *et al.* (1995b)
IL10	1	–	+	–	+	Gazzinelli *et al.* (1996); Kühn *et al.* (1993)
IL12	6	–	–	–	(+)	Mattner *et al.* (1996)
Rag1/Rag2	2	–	–	–	+	Mombaerts *et al.* (1992)
Selp	1	–	–	–	(+)	Bullard *et al.* (1996); Mayadas *et al.* (1993)
Tap1	17	–	–	–	(+)	van Kaer *et al.* (1992)
TgN(Bcl2)22Wehi	?	+	+	–	–	Strasser *et al.* (1991)
TgN(Lck Il4)1315Dbl	?	–	+	–	+	Lewis *et al.* (1993)

*See footnote to Table 4.

embryo-producing capacity. In addition, monitoring means that the risk to other colonies within the animal facility can be better calculated and precautions initiated.

Specified pathogen-free barrier units

By definition according to the international conventions (see pages 117–121, Box 1 and Table 3), specified pathogens cannot be found in a SPF barrier unit. However, no statement on the residual microbiological status is given, implying the possibility of extensive differences from one SPF colony to another (Heine, 1980; O'Rourke *et al.*, 1988; Boot *et al.*, 1996; Rodrigue and Lavoie, 1996). When transferring animals from one SPF to another SPF unit, it should be taken into account that by this action other 'non-pathogenic' microbes or variants may be introduced into the colony which can disturb the microbiological equilibrium, especially in immunodeficient animals (Oshugi *et al.*, 1996).

The SPF level can be established in units of very different size – individually ventilated cages, isolators, or a larger room unit within an animal facility – and it can be run within a certain scale of restriction. In the following we describe the highest standard of SPF, as required for an SPF-breeding unit. A closed area with a strict hygiene barrier system with respect to air supply, materials, food, bedding and personnel (Otis and Foster, 1983; ILAR Committee on Immunologically Compromised Rodents, 1989) is required. After disinfection of the SPF area, high-standard animals can be introduced either directly from a germ-free or gnotobiotic isolator or, when coming from an extramural source, via a mini-isolator (e.g. HAN-Gnotocage) where the filters have been sealed by a foil for the duration of the transfer through a peracitic acid, hydrogen peroxide or otherwise disinfected lock.

A *standardized diet* can be sterilized by X-ray irradiation or by autoclaving. In the case of X-ray irradiation the outside of the package has to be disinfected. If food is sterilized by autoclaving, it has to be 'fortified', i.e. heat labile vitamins have to be added in such an excess that sufficient amounts remain intact after heat treatment. When changing to a new batch, the hardness and the acceptance of the food after autoclaving should be regularly controlled. It should be mentioned that deviations from batch to batch cannot be avoided because of the naturally varying origin of the food ingredients.

Drinking water should be sterilized by heat, filtration ultraviolet (UV) light treatment, but without further precautions bacterial growth is still very rapid in the bottles and also in automatic drinking systems. Therefore, acid (e.g. hydrochloric or acetic acid) should be added to a pH of 3.0–2.5, which will inhibit the growth of microbes, including that of *Pseudomonas* spp. (ILAR Committee on Immunologically Compromised Rodents, 1989). One should note that acidified water may raise problems when vitamins or drugs are to be added. While acidification may change immune functions only marginally, extensive chlorination has been reported to alter the immune response (Fidler, 1997; Herman *et al.*, 1982).

Bedding should be dust-free (<1% dust) and autoclaved after one or two cycles of vacuum/steam exchange. Pregnant females, especially of poorly breeding strains, should be provided with additional nesting material such as autoclaved cellulose towels or nestlets (Sherwin, 1997; Van de Weerd *et al.*, 1997). The recommendations regarding the population density (Weihe, 1978) and the maintenance of biological rhythm (Wollnick, 1989) should be followed.

The microbiological status is to be regularly monitored, sick animals should be removed from the unit and submitted to necropsy/microbiological examination, and sentinels should be regularly checked (see pages 115–117). Single rooms should be emptied, sealed from the remaining unit, cleaned and then disinfected (e.g. with formalin, hydrogen peroxide or commercially available disinfectants), once or twice a year.

The highest risk for the system is, however, the *personnel* entering the barrier unit. They should be well trained (FELASA, 1995) and aware of hygiene risks. The members of the SPF-area staff should be as constant as possible. If staff members have come into contact with rodents outside the SPF area they should not be allowed to enter it until a certain period of time (4–7 days) has elapsed. Members of the staff may be checked regularly, especially after having had an infection (throat and stool specimen). Persons entering the SPF area should shower and wear sterilized clothing inside the barrier system. It must be stressed that within the SPF area strict

Box 2 Double-lock room

For SPF-containment:

1. Stationary position: room and lock doors are closed.
2. Place provisions from the clean floor in the lock; the animal caretaker enters the lock and closes the door.
3. Flush the lock.
4. Opening to the animal room: after closing the inner lock door, flush or disinfect the lock.
5. Working in the room.
6. Opening the inner lock door to the dirty corridor: waste is placed in the lock; the animal caretaker enters the lock and closes the door.
7. Flush the lock.
8. The door to the dirty corridor can now be opened.
9. After closing the door, flush or disinfect the lock.
10. Stationary position.

For experimental use:

Especially for experiments with infectious agents in immunocompromised animals.

hygiene rules must be followed. At least once a week the floor and all equipment should be washed down with a non-volatile formulation of a disinfectant.

If properly managed, such systems may stay 'clean' for many years, although the permanent risk from personnel and technical accidents should not be neglected. In addition, it should be realized that an outbreak of an infection is unlikely to be restricted to a single room (Boot *et al.*, 1996). Therefore, one-way direction animal rooms have been proposed, equipped with a lock to the clean corridor and a lock to the dirty one (see Box 2).

Laminar air flow cabinets

In this design a constant flow of HEPA-filtered air of at least 1.2 f s^{-1} (0.4 m s^{-1}) has to be achieved by mass air displacement within the space to be used for setting up the animal cages (ILAR Committee on Immunologically Compromised Rodents, 1989). Each hindrance within the space, e.g. the cages themselves, may induce whirls and counterflow, which increase the risk of contamination (Thigpen and Ross, 1983). For this reason, exclusively filter covered cages should be used, which protect the animals from cross-contamination within the flow and, in addition, allow protected transfer to a working bench for changing of cages and for experimental manipulation. For low-risk infection experiments, the suckling version may be used. Altogether this housing system is not economical and should only be used in exceptional cases.

Ventilated cabinets

These filter-equipped units, optionally equipped also with a climatization facility, are used to protect small rodents from contamination in the room, and personnel from exposure to dust, allergens, microbes and emissions from the animals (the latter if linked to the exhaust from the room). The uncontrolled status that occurs when doors are opened can be avoided by using filter-covered cages, which will, in addition, protect the animals against cross-contamination within the cabinet and during transmission to the working bench. As in the mass air displacement system, the high airflow over the filter top will prevent bad climatic conditions within the cage. The same equipment switched to negative pressure offers considerable protection in animal experiments involving infectious agents.

Individually ventilated cages

Principal considerations

Microisolator cages (Kraft, 1958; Serrano, 1971) combine the advantages, especially when working with transgenic and immunodeficient strains, of accessibility and isolation at the cage level, and are discussed in more detail below. The original problem encountered with isolator cages was to

combine sufficient ventilation with effective filtration (Lipman, 1992). In fact, it was found that in static (i.e. not forced) ventilation conditions the exchange of air between the interior of the cage and the room was fairly low, causing the humidity and the carbon dioxide and ammonia concentrations to increase to intolerable levels within the cage (Schoeb *et al.*, 1982; Lipman *et al.*, 1992; Choi *et al.*, 1994; Huercamp and Lehner, 1994). In addition, residual air exchange was found to occur mostly via the space between the filter top cover and the cage (Keller *et al.*, 1989), implying a break in the petri-dish barrier. Therefore, animals should not be housed in filter top cages in static conditions other than for short-term transport within a facility. A considerable improvement was achieved by the use of individually ventilated cages (IVC) system. Various systems of this type are now commercially available as a complete rack unit with HEPA-filtered ventilation and exhaust (for a comparison of different systems see Corning and Lipman, 1991; Hasenau *et al.*, 1993; Perkins and Lipman, 1996; Tu *et al.*, 1997). In the IVC system, the environmental conditions within the cages are less variable and less stressful for the animals. Temperature, humidity and ammonia levels comply with or are better than those required by the *Code of Practice for Housing and Care of Animals used in Scientific Procedures* (Home Office, 1989). Although the noise levels are higher than the room background level, they are found to be tolerable (Perkins and Lipman, 1996).

The major advantage of the IVC system with regard to immunodeficient animals is the protection of the animals from airborne contamination at the cage level. We and others have found that mice can be protected against MHV infection by positive-pressure IVCs within a room or rack that is also occupied by infected mice in unprotected cages (Dillehay *et al.*, 1990; Lipman *et al.*, 1993). In addition to protecting animals, the IVC system also reduces the levels of aeroallergens, which may cause health problems for personnel (Hunskaar and Fosse, 1993; Clough *et al.*, 1995) and eliminates pollutants if connected to the exhaust. However, it should be mentioned that, if not properly constructed, the exhaust of the cages can soil channels and obstruct prefilters.

Commercially available IVC-racks are equipped with a ventilation unit that is mounted on the top, on the bottom or separately in the animal room. A further development could be the integration of the IVC system into the room ventilation, allowing ease of accessibility to the machinery. The air supply to the residual room does not need to be ultrafiltered and the air exchange rate can be reduced, saving costs by up to 50% and thus compensating, at least partially, for the high cost of the IVC system (Lipman, 1993; Clough *et al.*, 1995). In the latter context it should be remembered that the animals are exposed directly to fluctuations, especially in the temperature of the climatization machinery, without any compensation from the air of the room.

Running the IVC system at negative pressure helps to protect the environment from contamination by quarantined or infected animals. However, special requirements need to be considered when working with immunocompromised animals (see pages 137–138).

Handling

This is the most critical and most underestimated part of running an IVC system. Principally, three different hygiene levels have to be distinguished: (1) the high sterility level of autoclaved material, diet and water; (2) the room and the outside of the cage; and (3) the inside of the cage, i.e. the animals and their immediate environment. The latter may be different from cage to cage. With regard to manipulation, the protocol in Box 3 is recommended (Homberger, personal communication).

Box 3 Handling of IVCs

1. The laminar flow bench, class II (Biohazard), is switched on.
2. Dilute the sterilization compound (disinfectant)* for gloves. Gloves must be kept moist during the whole procedure.
3. Place an autoclaved filter top cage in the flow bench.
4. The cage to be changed is placed in the bench.
5. The filter tops of both cages are removed and set aside.
6. Sterile diet and water bottle are placed in the clean cage.
7. The animals are transferred to the new cage by using a sterilized forceps.
8. The filter tops are replaced and the cages are removed from the bench.
9. The bench is disinfected occasionally.
10. In the case of infectious animals, the used cage is autoclaved.

* *Recommendation:* Use a very fast-acting sterilization compound, e.g. one based on glutardialdehyde (Chlidox) or chlordioxide (e.g. Chlidox or Alcide). Alcohol and commercially available hand disinfectants are not sufficient.

The procedure is very labour intensive, but this can be compensated for in part by extending the cage changing interval (due to the high ventilation rate, bedding is kept dry and the ammonia level low). Increasing the change interval reduces the stress on the animals. An automatic watering system saves time, but carries a higher risk of contamination due to the interconnection of individual cages and bacterial growth in the pipes.

The IVC system, although expensive to establish and time consuming to run can be used to breed and maintain animals in SPF conditions, and is particularly useful when the structural prerequisites for an SPF unit are lacking and easy access is indispensible for experimental reasons. The negative-pressure version of the IVC system is ideal as a quarantine unit for animals received from different sources and for experiments involving low pathogenicity micro-organisms.

Isolators

Isolators in the positive-pressure version are indispensible for germ-free or gnotobiotic stocks, and in the negative-pressure version as a quarantine station or for high-risk infection experiments. Isolators are made of

flexible polyvinyl film, polycarbonate or stainless steel, the latter two being physically more robust than the first.

In the positive-pressure version, the air supply is equipped with an autoclavable HEPA-filter unit and an exhaust with a valve to prevent contamination due to backflow, or a further HEPA-filter unit. A chemically sterilizable lock is used to connect the interior of the isolator to a supply chamber (Trexler, 1983). Depending on the construction, chemical sterilization of the interconnecting space is required, and this has to be flushed with air from the isolator directly to the exhaust by a connecting tube. Materials are autoclaved in loose packaging within the supply chamber, and the water bottles (screw top, semi-stopped) are sterilized and cooled in the autoclave below the seeding point. Each autoclaving process should be controlled in the supply chamber by means of temperature indicators (paper and/or maxima-thermometer) for immediate validation, and by using a bio-indicator (*Bacillus stearothermophilus*) for the retrograde validation. It should be mentioned that there is some retardation of the heating within the supply chamber with respect to the autoclave chamber, which has to be compensated for by the sterilization process.

Dietary problems may result from the considerable reduction of the nutritional value after thermal sterilization – the latter should be sufficient to kill bacterial spores. Alternatively, an X-ray irradiated diet or, for special investigations (e.g. endotoxin effects in germ-free animals), an X-ray irradiated semi-synthetic diet (Enss *et al.*, 1997) can be used after chemical sterilization of the outside of the package.

In the negative-pressure version, the exhaust air is passed through a HEPA-filter and the lock is used together with the autoclaving chamber for the removal and treatment of waste. This system is used for experiments involving high-risk pathogens.

A combination of both versions, where the inlet and exhaust air are HEPA-filtered, offers the protection of the environment and of the animals. If the protection of the environment is of primary importance, the isolator should be run with an overall negative pressure. The lock and the autoclaving chamber are used for both the sterile supply and the disinfection of waste. The most serious disadvantages of this system are that it is extremely labour-intensive and the difficulty of manipulating the animals within the isolator.

Special Considerations on Immunocompromised Animals

Propagation

The consequences of gene manipulation on susceptibility to disease cannot be predicted fully (e.g. Fernandez-Salguero *et al.*, 1995). Therefore, the aim when creating a gene-manipulated animal should be to maintain the highest possible standards of hygiene, especially during the second part of the procedure, when the foster mothers and the embryos to be reimplanted are being handled, and during the further management of the colonies. Of course this is of special importance when raising immunocompromised animals. In practice, special staff should be available for these tasks. In

addition, different people should be responsible for the manipulation and experimentation on the low restricted side and on the clean side, respectively. The risk of contaminating the clean side via the embryos is low if proper washing is carried out (see page 163).

Adherence to a strict regimen offers the possibility of raising transgenic animals at a level of hygiene that is adequate for immunocompromised strains, thus avoiding time-consuming rederivation. In this context, it should be mentioned that a clear-cut designation of the donor strain, the construct and the ES cell line (if used) should be given according to international rules (Davisson, 1996).

Housing

The microbiological standards outlined above are of special importance for immunodeficient animals because of their high susceptibility to common as well as opportunistic infections (Mossmann, 1992). Immunocompetent animals are able to overcome most infections owing to their immune system; many immunocompromised lines, however, are unable to eliminate the pathogens (Rozengurt and Sanchez, 1993). In addition, when working with immunodeficient animals attention should be directed to the translocation of bacteria from the gastrointestinal tract through the epithelial mucosa and into the organism (Oshugi *et al.*, 1996). The question arises as to whether the SPF standard is adequate for severely immunodeficient animals, or if a more stringent contaminant standard (gnotobiotic, germ free) is necessary. However, it should be taken into account that the immune response may depend on the general prestimulation of the immune system, which is lower in gnotobiotic or germ-free animals. Therefore, caution should be exercised when comparing experimental results obtained in animals maintained at different hygiene levels.

Experiments with infectious agents

Immunocompromised animals in particular should be protected from the environment and, at the same time, the environment should be protected from the infectious agent used in the experiment. As already mentioned, ventilated cabinets equipped with filter hood cages offer a far-reaching solution, but cannot satisfy both these functions unless they are used in combination with a barrier system. The development of sealed IVCs, i.e. biocontainers at the cage level, offers a further improvement in this direction. The highest standard solution available is the use of isolators in the combined version (see page 137).

Mating Systems

As mentioned earlier, the phenotype of a gene governing a state of immunodeficiency – either 'natural', induced or transgenic – may be seriously altered by its genetic background. While most of the established natural and induced mutants have been established in or transferred to an inbred background, many of the most recently developed transgenic and

targeted mutants have a segregated mixed background, which should be back-crossed to more than one defined inbred strain in order to be able to make comparisons with the transgenic or targeted mutant and the modulating effects of different genetic backgrounds. There are many mating systems for breeding rodents (Green, 1981). Unfortunately not all of them can be used either to propagate or to maintain a mutation resulting in immunodeficiency in the bearer. Nevertheless, the major mating systems are: (1) inbreeding by brother × sister matings, thus transferring the mutant/mutated allele to a standard inbred strain background; and (2) propagating the mutation without inbreeding. The latter is used only in those cases where inbreeding is not successful. Assisted reproduction such as *in vitro* fertilization (IVF; Box 4) with embryo transfer (Box 5) might be required. If viability and fertility are reduced, specific measures might be necessary. If the mutant females are unable to mate, or, although being fertile in the sense that they are able to produce functional oocytes are infertile in the sense that they are physically unable to bring offspring to term (as is the case in mice carrying the obese mutation or muscular dystrophy), transplantation of ovaries to unaffected syngeneic or, for example, C.B. 17-*scid* females (Stevens, 1957).

Box 4 In vitro *fertilization of mouse ova*

The protocol described below is a modification of the one reported by Hogan *et al.* (1994).

- *Animals:* 6- to 8-week-old females (superovulated); fertile males.
- *Media:* PB1 (Whittingham, 1971) for embryo collection; Whitten's medium (Whitten, 1971) for culture.

1. Cover Whitten's medium with paraffin oil, preincubate overnight (37°C, 55% CO_2, 95% humidity).
2. 12.0 h after injection of the females with human chorionic gonadotrophin (hCG), kill the males and collect sperm from the epididymis. Suspend sperm in Whitten's medium and incubate for 1.5 h.
3. 12.5 h after injection of the females with hCG, kill the females and collect oocytes in PB1 without removing the cumulus cell mass. Incubate oocytes in Whitten's medium for 1 h.
4. 13.5 h after injection of the females with hCG, add the oocytes to the sperm suspension.
5. 4 h after adding the oocytes, change the medium.

Box 5 Embryo transfer to the oviducts of pseudopregnant surrogate dams

Pseudopregnancy is induced by mating females with vasectomized males of proven sterility, or genetically sterile males, heterozygous for the T(X;16) 16H reciprocal translocation indicated by the Tabby (*Ta*) marker (Lyon *et al.*, 1964), or heterozygous in two pseudo-allelic

(*cont.*)

Box 5 (*cont.*)

variants of the mouse *t* complex (T/tw^2) (Silver, 1985). Females selected to be in oestrus will increase the yield of recipients. Note that, in contrast to mice, it is difficult to produce timed pseudopregnant surrogate rats – neither cervical stimulation with a vibrator nor the application of a vaginal tampon has been very effective. However, vasectomized male rats have provided reasonable results. Copulatory plugs are easy to determine (in rats by means of an otoscope). Induction of pseudopregnancy is timed such that a synchronous (i.e. same chronological stage) or asynchronous (i.e. recipient stage minus 1 day of embryo development) transfer can be performed.

Although embryo transfer may be performed by one operator, two are recommended, one to anaesthetize the recipient, exteriorize the oviduct and the uterine horn, and close the abdominal wound (recipient operator), and the other to load the transfer pipette with the embryos and perform the actual transfer (donor operator). This type of procedure will cause only minimal distress to the recipient female and, therefore, attain much better results.

All cleavage stages from zygotes to morulae can be transferred to the oviduct of pseudopregnant recipients to complete their development with a high rate of success. The timing of pseudopregnancy and developmental stage of the embryo is less critical than in the case of uterine transfers. Best results are achieved if 2- to 8-cell embryos are transferred. With this technique the embryos must have an intact zona pellucida. Normally only unilateral transfers are performed with 5–8 embryos per recipient. If more than 8 embryos are to be transferred to one recipient, they are partitioned and transferred to both oviducts.

The surrogate dam is anaesthetized. After hair clipping and wiping the recipient's lower back with 70% ethanol, the animal is placed under a stereomicroscope with strong incident illumination. An incision about 1 cm in length is made at the level of the ovaries. The skin is slid to either side toward the location of the ovary. The body wall is severed with fine scissors. The ovary, oviduct and proximal end of the uterine horn are exteriorized and fixed with a microwire clamp. The ovarian capsule is then disrupted by means of two No. 5 micro-forceps. To avoid excessive haemorrhage from ruptured vessels (a regular occurrence in rats) one drop of epinephrine solution is applied topically to the bursa ovarica. The infundibulum (always pointing caudally) is located, the transfer capillary inserted and fixed in place with No. 5 micro-forceps, and the embryos deposited by carefully blowing into the mouthpiece. The 2–3 air bubbles drawn up into the pipette to disrupt capillary suction before loading with embryos now serve as an indicator of the amount of medium containing embryos that has been ejected. Withdrawing the capillary the infundibulum should be compressed with the micro-forceps to prevent any reflux and loss of embryos. All exteriorized organs are replaced and the skin incision is closed with wound clips. No suturing of the muscle incision is necessary, providing it has been kept small.

Inbreeding

A unique advantage in working with mice and rats is the availability of standard inbred strains. By using this type of strain, including an F1 hybrid, rather than an outbred stock or a stock with a mixed genetic background, it is possible to eliminate genetic variability as a source of variation. This homogeneity within strains is obtained by continuous brother × sister (B × S), or younger parent × offspring matings for a minimum of 20 generations. After this period, 98.02% of all loci within the genome of either animal of the particular strain should be homozygous. After F12, the remaining heterozygosity within the (incipient) inbred strain will decrease by 19.1% per generation. The increase in homozygosity deviates from the expected value if there is any selective force (inadvertent or intended) towards a certain phenotype or mutation.

Congenic strains

To identify the effects of a particular locus, the use of congenic strains is obligatory. Congenic animals represent attempts of genetic identity with the inbred partner strain, except for the alleles at a single locus. Congenic strains can be produced by certain mating systems, depending on the nature of the differentiating locus, i.e. whether the phenotype can be determined in the heterozygous state and whether the locus affects viability or fertility. Furthermore, as the phenotype of a gene may be altered by other genes of the genetic background, it might be advisable to transfer the variant or mutated allele onto a further standard inbred strain. A detailed description and analysis of the various systems of transferring a mutation/mutated gene onto an inbred background has been presented by Green (1981).

Most of the targeted mutations are induced in embryonic stem (ES) cells, derived from a 129 strain, which are then injected into C57BL/6 blastocysts with a subsequent mating to C57BL/6 followed by B × S or parent × offspring matings. This will result in strains that might be considered as recombinant congenic with an unknown admixture of finally fixed alleles (≥ F20) of the two progenitor strains. It is therefore advisable to propagate the targeted mutation further by back-crossing to C57BL/6 or 129, or another common inbred strain.

If ES cells derived from C57BL/6 or BALB/c are used and targeted ES cells are injected into the blastocysts of the corresponding progenitor strain, at least two cycles of back-crossing should be performed to compensate for possible chromosomal defects acquired by the ES cell line during *in vitro* culture.

The simplest approach for generating a congenic strain is to produce an F1 hybrid from a cross between an animal carrying the allele of interest with the selected inbred partner. The resulting progeny are back-crossed to the inbred partner. This is repeated at least for a further nine back-cross generations. With this scheme, one-half of the unwanted donor genome not linked to the differentiating locus is lost at every generation. Since the advent of PCR-typed DNA-markers which enable identification of the

locus of interest or one closely linked to it, other mating systems are no longer required. If the recessive allele in the homozygous state is lethal or induces sterility, a known heterozygote (as defined by genotyping) is back-crossed to the selected inbred strain. If genotyping is not possible, cross-intercross matings are performed, whereby carriers are identified by the production of mutant offspring. Once identified, the homozygote is crossed with the inbred strain and the resultant progeny are again intercrossed.

There is a statistical probability that mice from an incipient congenic strain developed by using the back-cross system continue to segregate in loci derived from the (induced) mutant donor origin (Fig. 1). The amount of residual genome retained (differential segment) depends on crossing-over events near the locus of interest and thus on the number of back-crosses performed. The probability (P_n) of a contaminant gene of donor origin other than the differentiating gene can be calculated as $P_n = (1-c)^{N-1}$, where N is the number of back-cross generations ($N = 1$ = F1) and c is the probability of crossing over between the differential and residual passenger genes. When $c = 0.5$, i.e. when the passenger gene is not linked to the differential locus, the probability of retaining such an allele is 0.002 after 10 generations of back-crossing. However, if linked genes are considered, one has to expect that after 10 generations the probability of retaining an allele of the donor strain within the 20 cM range is about 13%, within the 10 cM range about 39%. For 'speed congenic' production see

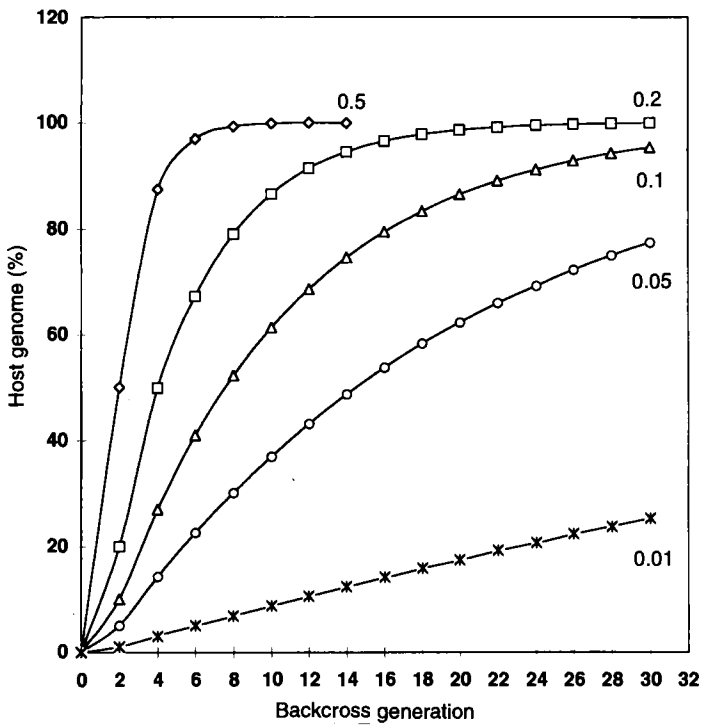


Figure 1. Probability that a certain percentage of host genome has become fixed at various back-cross generations for genes that are unlinked to the differentiating locus ($c = 0.5$), are moderately linked ($c = 0.2$ to 0.005) or tightly linked ($c = 0.01$).

Wakeland *et al.* (in press), who have used computer simulation to model various strategies.

By applying marker-assisted selection protocols, i.e. a genome-wide scan of genetic polymorphisms distinguishing donor and background strain, the production of genetically defined congenic strains is possible within a period of 1.5–2 years. Apparently, with low density marker spacing (25 cM apart) and screening of four litters (only males) at every generation a sufficient back-crossing is achieved after only five generations of back-crossing. In addition, the genome scan allows to identify the chromosomal location of a transgene in *N*2 and does provide information on (unwanted) donor-derived regions.

Propagation without inbreeding

Certain mutants cannot successfully be inbred or transferred to a specific inbred background. In these cases the mutation has to be maintained on a hybrid background such as an outbred stock, or descendants of an F1 hybrid. It is thought that these animals with a heterogeneous background are hardier, more productive, faster growing and have a longer life-expectancy. For example, it is extremely difficult to maintain the athymic nude mutation of the rat (*Hfh11*^{rnu}, *Hfh11*$^{rnu\text{-}N}$) on DA and LEW backgrounds.

Many of the targeted mutants are maintained on the variable, mixed background of the ES-cell donor and recipient strain and sometimes another 'prolific' strain or stock. If an immunological mutant cannot successfully be inbred due to effects on viability and fertility, there is no other option but to maintain it on a segregating background or to back-cross the mutant permanently to two different standard inbred strains and to produce homozygous mutant offspring on an F1 background by mating mutant-bearing animals of either strain.

In all instances where research is to be carried out using animals from partially inbred or back-crossed strains or from non-inbred stocks, one should be aware of the genetic variability of these experimental animals and therefore use as controls unaffected (heterozygous and +/+) littermates. If these littermates are not available, F2 offspring derived from the two progenitor genomes provide the closest approximation to the background genotype; F1 hybrids, being genotypically identical, will be the least suitable match.

Genetic Monitoring

Mutations and differential fixation of alleles at early generations of inbreeding may alter the genetic constitution and thus the phenotype of an inbred strain. Many of the phenotypic differences encountered between substrains are due to these factors. Inadvertent outcrossing (genetic contamination) will alter a strain seriously, making its further use for research questionable, since the results are no longer comparable and

repeatable. It is therefore of utmost importance to separate strains that are not immediately to be distinguished by their phenotypic appearance. If, however, due to shortage of shelf space and separate animal rooms one has to co-maintain several strains in one room, regular screenings for the mutant as well as strain-discriminating markers are indispensable.

Proper colony management is the first step towards the provision of authentic laboratory animals (Box 6).

Box 6 Principles of proper colony management

1. During regular handling only one cage at a time should be managed. This will prevent accidental exchange of animals from different cages.
2. Animals that have escaped or dropped to the floor must never be returned to the suspected cage unless the animal can be identified by a non-interchangeable sign such as an ear tag, a tattoo or a transponder signal specifying the animal by strain name (code) and animal number. Traps should be set in larger animal rooms to catch stray animals. Animals caught outside the cage should be killed or isolated.
3. Cages and hoods should be in sufficient condition that no animal can escape or enter another cage, a problem more often encountered in mouse than in rat breeding units.
4. For ease of identification and in order to prevent an inadvertent mix-up, cage tags should have a strain-specific colour code and a strain-specific number (code).
5. Cage tags should always be filled out properly, including the strain name, strain number, parentage, date of birth and generation.
6. If a cage tag is lost, one should not redefine the cage except in the case of definite proof of identity through marked animals within the cage.
7. If at weaning the number of animals is larger than that recorded at birth the whole litter should be discarded or submitted to the genetic monitoring laboratory.
8. If it is inevitable that several strains are housed together, care should be taken to select strains that are easy to distinguish by their coat colour and that will give rise to hybrid offspring different in pigmentation to either parental strain. Strains or substrains that are difficult to differentiate not only by phenotypic appearance but also by laboratory tests must not be kept in the same quarters.
9. Any change in phenotype and/or increase in productivity should immediately be reported to the colony supervisor. The latter change should always be considered suspect for a possible genetic contamination.
10. Regular training programmes on basic Mendelian genetics, systems of mating and the reproductive physiology of the animals maintained should make animal technicians and caretakers conscious of the consequences any mistake will impose on the colonies. Further training should stress the importance of a search for deviants as potentially new models for biomedical research.

As repeated handling of animals during regular caretaking cannot be avoided, there is always the risk of errors. An animal might inadvertently be placed in the wrong cage, or an incorrect entry put on the label. Assigning this type of work to well-trained and highly motivated animal technicians should be a matter of course. The colony set-up and the structuring of nucleus colonies in a single (Festing, 1979) or parallel-modified line system (Hedrich, 1990), pedigreed expansion colonies and multiplication colonies should be self-evident, and strictly monitored. There are several publications dealing with the set-up of colonies for maintenance and large-scale production (Green, 1966; Lane-Petter and Pearson, 1971; Hansen *et al.*, 1973; Festing, 1979). In general, permanent monogamous mating is to be given preference, as this provides a constant colony output with minimal disturbance of the litters during the early postnatal period and by utilizing the chance that females are inseminated at the post-partum oestrus.

The measures required for genotyping a strain have to be adjusted to the specific needs and may depend on the scientific purpose, and on the physical maintenance conditions and laboratory equipment used. Nevertheless, there are specific demands (although unfortunately not stringent rules) on how to authenticate a strain or to verify its integrity.

For any authentication it is necessary to determine a genetic profile that is to be compared with published data (if available), and which allows one to distinguish between (all) strains/stocks maintained in one unit. In general, this profile is composed of monogenetic polymorphic markers, which may be further differentiated by the method of detection into immunological, biochemical, cytogenetical, morphological and DNA markers. Due to the recent rapid development of microsatellite markers (simple tandem repeats (STRs)), these have almost fully replaced the classical genetic markers in routine applications. A large number of primer pairs for mice and rats is available (e.g. through Research Genetics Inc., Huntsville, AL, USA; http://www.resgen.com). Other sources of primers are also available through the World Wide Web (e.g. markers developed by the Wellcome Trust Centre for Human Genetics, Oxford, UK; ftp://ftp.well.ox.ac.uk/pub/genetics/ratmap). However, as with the classical markers it is indispensable to set up a genetic profile representing a random sample of the genome, which should be evenly spaced on the chromosomes, and which will allow one to discriminate between all strains maintained per separate housing unit. Unfortunately this information is only partially available and not yet compiled in an accessible database. Conditions for PCR amplification and electrophoretic separation of the amplicons are described in Box 7. These conditions deviate from those provided primarily with respect to electrophoretic separation. As there is only one amplification protocol it could be necessary to adjust the temperature conditions and Mg^{2+} concentration for each microsatellite marker. For routine screening, separation on agarose gel and visualization by ethidium bromide will suffice. If separation of the amplicons is insufficient in agarose, one should run a polyacrylamide gel electrophoresis (PAGE; see Box 7). As radioactive labelling is with ^{32}P using a kinase reaction, and since the isotope half-life is relatively short, a silver staining procedure is recommended (Box 7).

Box 7 Protocol for characterizing and typing STRs with one primer set per PCR reaction

The amounts needed are based on the quantities calculated for a single reaction well plus a small excess. To work out the amount of the various reaction mixtures, simply multiply by the number of DNA samples to be tested:

1. Add mineral oil (30 µl) to each well of a 96-well plate (not necessary when using hot bonnet).
2. Pipette 5 µl template DNA (20 ng μl^{-1}) into each well.
3. Centrifuge the plate briefly to collect the template in the bottom of the wells; apply the plate at 96°C for 3 min, and then at 4°C until adding the mastermix.

PCR:

1. Preparation of mastermix:

Forward primer (6.7 µM)	0.25 µl
Reverse primer (6.7 µM)	0.25 µl
dNTP (1.25 mM/dNTP)	2.5 µl
10× PCR buffer	1.5 µl
H_2O	4.75 µl
Taq-polymerase (5 U μl^{-1})	0.15 µl
Total	10.00 µl.

2. Transfer 10 µl mastermix to each well.
3. Centrifuge and run the PCR reaction.
4. Add 3 µl BFB to each well.
5. Load on a 3% Nu sieve or a 1.5% Sigma Type II Agarose gel in 1 × TBE; alternatively, use polyacrylamide gel (PAGE) (see below).
6. Run the electrophoresis for 3–4 h at 70 V.
7. Stain with ethidium bromide (in case of PAGE, use silver staining; see below)

PCR programme

1. 3 min at 94°C 1×
2. 15 s at 94°C
3. 1 min at 55°C } 30×
4. 2 min at 72°C
5. 7 min at 72°C 1×

PAGE:

- 6–7.5% polyacrylamide/bis(acrylamide) in 1× TBE (13 × 16 cm) (Sambrook *et al.*, 1989).
- Spacer 0.4 mm.
- Probe volume 2–6 µl (gel loading buffer type 2 (Sambrook *et al.*, 1989).

(*cont.*)

Box 7 (*cont.*)

Silver staining:

Note: Use only twice-distilled water for all solutions and washings and prepare all solutions directly before use.

- Fixing: 30 min, 10% acetic acid (v/v); 3 × 2-min wash in H_2O bidest.
- Staining: 20–30 min, 0.1% $AgNO_3$ (w/v), 0.037% formaldehyde. (The time of staining depends on the gel concentration and gel thickness.) Rinse for 5–10 s with H_2O bidest.
- Developing: 2–15 min in 2.5% Na_2CO_3; add 0.037% formaldehyde; add 0.002% sodium thiosulphate (solution). (These solutions must have a temperature of 4–5°C, maximum 10°C, and pH 12.0. *Note:* Do not use any plastic container for developing procedure.)
- Desilvering: in 10% acetic acid.

Nevertheless, the classical markers are still relevant and may need to be verified. They may even allow for a faster and less expensive phenotyping.

Immunological markers

Immunological markers comprise cell surface markers, such as: major histocompatibility antigens (*H2* in the mouse and *RT1* in the rat); lymphocyte differentiation antigens; red blood cell antigens; minor histocompatibility antigens; allotypes (immunoglobulin heavy-chain variants), which can be determined by Trypan blue dye exclusion test (see pages 189–204); flow cytometry (see pages 23–57); immunodiffusion, ELISA (see pages 621–650); and immunohistochemistry ELISA (see pages 257–286), using specific antibodies. The availability of antibodies depends on the specific marker and the species, with a broader spectrum available for mice. These markers may also be demonstrated by applying molecular biology techniques, such as oligotyping of major histocompatibility complex (MHC) class I and class II genes by reverse transcriptase PCR (RT-PCR) and dot blot and reverse dot blot hybridization, respectively (see pages 148–149; for other specific markers check also: http://www.informatics.jax.org/mgd.html).

Biochemical markers

These are the classical electrophoretic markers, which almost have been replaced by STR typing unless a specific allelic expression is to be verified in an experiment. Apart from qualitative enzyme and protein polymorphisms, enzyme differences, such as Car2 in mice, may have to be quantified to differentiate between homozygous *Car2°/Car2°*, heterozygous *Car2°/+* and wild-type (+/+) mice.

Box 8 Identification of MHC class I and II alleles by oligotyping

***H 2D* alleles of laboratory mice**

The homologies of the MHC class I genes in the murine species do not allow identification of each *H2* class I allele using one specific oligonucleotide. Three allele-specific oligonucleotides are required to identify one of the *H2D* alleles. Two of these oligonucleotides are used as a specific pair of primers to predifferentiate the alleles, independently of the gene loci by enzymatic amplification of a relevant RNA fragment. It is advisable to use a DNA polymerase that shows reverse transcriptase activities, as this will reduce the time and cost of typing. In the case of the *H2D* gene, the complementary sequences from the specific forward and backward primers are located in the highly polymorphic exons 2 and 3 of the class I mRNA, so that the resulting amplicons include further polymorphic areas (Fig. 2). The final identification is performed by hybridization of an amplified fragment with the third oligonucleotide that corresponds to an allele-specific sequence within the amplicon.

1. Isolate total RNA from tissue.
2. Predifferentiate *H2D* alleles by RT-PCR using allele-specific primer pairs. The optimal annealing temperature ($T_{A_{opt}}$) of the primer pairs should be calculated by the nearest neighbour method, which takes into consideration the enthalpy and entropy from each base pair (Rychlik and Roads, 1989; Rychlik *et al.*, 1990). It is recommended that 'touch down PCR' be run over all cycles in order to increase the stringency of the PCR conditions.
3. The amplified fragments are visualized by agarose gel electrophoresis and ethidium bromide staining.
4. Identify *H2D* alleles by dot blot hybridization using allele-specific oligonucleotides (Fig. 3). The dot blots are prepared by spotting an aliquot of the denaturated PCR products onto a nylon membrane. The DNA is immobilized by UV cross-linking or by baking the membrane.
5. The allele-specific oligonucleotides are labelled with radioactive or non-radioactive markers.
6. The hybridization procedure is carried out under absolute stringent conditions. The dissociation temperature (T_d) of oligonucleotides should be calculated by the nearest neighbour method, while the optimal hybridization temperature ($T_{hyb_{opt}}$) must be determined empirically. DNA/DNA hybrid detection depends on the type of oligo-labelling.

MHC class II RT1.B$_\alpha$ and RT1.B$_\beta$ alleles of inbred rat strains by oligotyping

The differentiation of the MHC class II alleles of laboratory rats is based on the same principles as the *H2D* typing. Prior to hybridization using specific oligonucleotides, a RT-PCR is needed to amplify the

relevant allele fragments. In contrast to the MHC class I genes, the non-orthologous MHC class II genes diverge clearly in their nucleotide sequences within a species (Wakeland *et al.*, 1990). For this reason the two *RT1.B* genes can be differentiated by RT-PCR using gene-specific primer pairs flanking the highly polymorphic exon 2. The final identification is followed by reverse dot blot hybridization of the amplicons. In this case the hybridization partners change their function. Compared to *H2D* typing the advantage of this procedure is that there is only a limited number of protocols because the hybridization temperature for each $RT1.B_{\alpha}$ or $RT1B_{\beta}$ allele is identical.

1. Isolate total RNA from tissue.
2. Differentiate the $RT1.B_{\alpha}$ and $RT1.B_{\beta}$ genes by RT-PCR using gene-specific primer pairs.
3. The optimal annealing temperature ($T_{A_{opt}}$) of the primer pairs should be calculated by the nearest neighbour method as for the predifferentiation of *H2D* alleles (Rychlik and Roads, 1989; Rychlik *et al.*, 1990). It is recommended that a 'touch down PCR' be run over all cycles to increase the stringency of the PCR conditions.
4. The amplicons should be labelled with Dig UTP during the PCR, to reduce the number of protocols.
5. The amplified fragments are visualized by agarose gel electrophoresis and ethidium bromide staining.
6. Identify the $RT1.B_{\alpha}$ and $RT1.B_{\beta}$ alleles by reverse dot blot hybridization using two sets of specific oligonucleotides for the $RT1.B_{\alpha}$ and $RT1.B_{\beta}$ alleles (Fig. 4).
7. The oligonucleotides should correspond to the hypervariable areas of exon 2 of the $RT1.B_{\alpha}$ and $RT1.B_{\beta}$ genes. It is important that all oligonucleotides of one set hybridize at about the same temperature.
8. The reverse dot blots are prepared by spotting the tailed oligo dT oligonucleotides onto a nylon membrane with a dot blot apparatus. The oligonucleotides are immobilized by UV cross-linking with the membrane.
9. The $RT1.B_{\alpha}$ or $RT1.B_{\beta}$ allele can be identified by its hybridization pattern (Fig. 4).

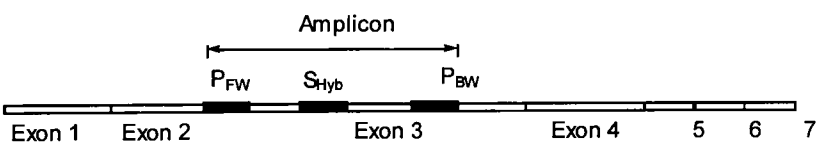


Figure 2. MHC class I mRNA. P_{FW}, forward primer; P_{BW}, backward primer; S_{hyb}, oligonucleotide.

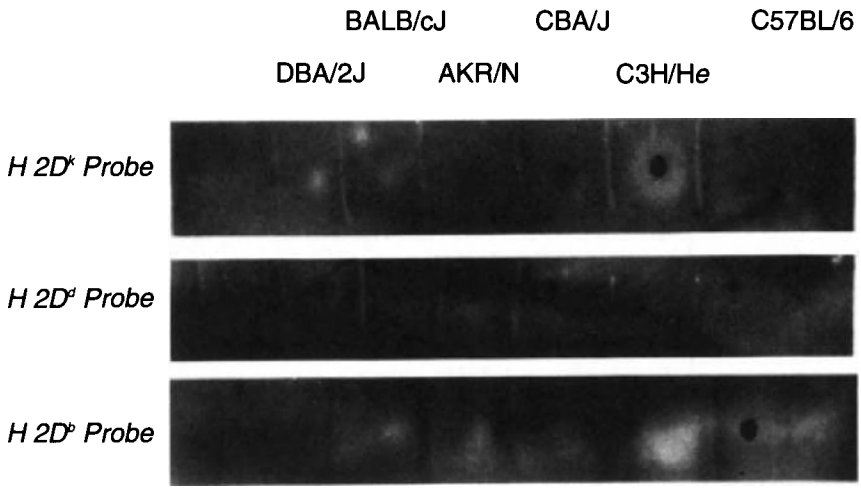
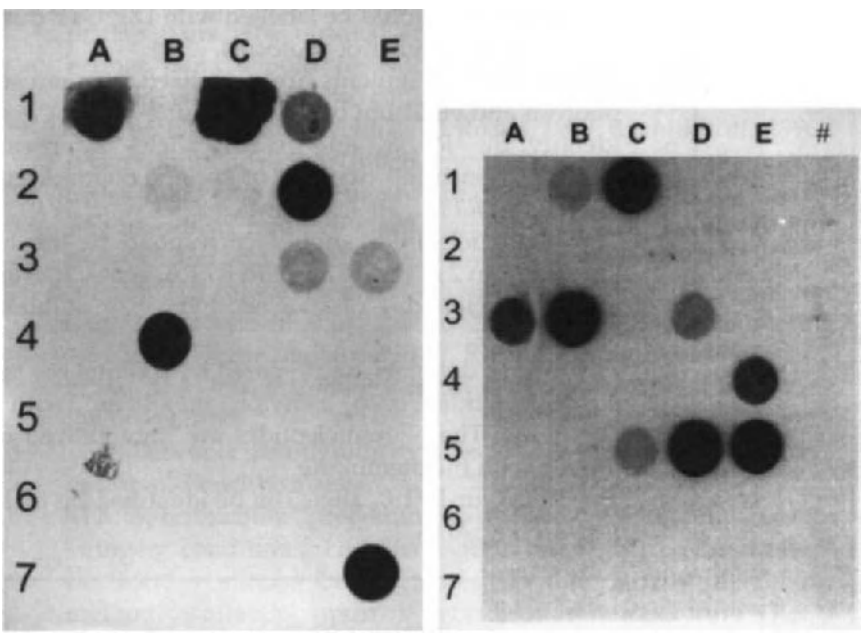


Figure 3. Identification of *H2D* alleles by RT-PCR and hybridization using allele-specific oligonucleotides.



(left) (right)

Figure 4. Identification of the $RT1.B_{\beta}^{u}$ (left) and $RT1.B_{\alpha}^{u}$ allele (right) by reverse dot blot hybridization. Columns A to E mark the variable areas in the exon 2 of the *RT1.B* gene. The possible oligonucleotides for each variable area are fixed on lines 1–7 of the membranes. (#) A column that contains only one oligonucleotide specific for a 6 bp insertion in $Rt1.B_{\beta}$ alleles.

Cytogenetic markers

These play a role if mice carrying numerical variants and structural aberrations of chromosomes are being maintained (e.g. the T(X;16)16H translocation; see page 139).

Morphological markers

These include coat colour and pelage variants, but also markers controlling skeletal abnormalities and metabolic and neurological deviants. A reasonable amount of coat colour genes show pleiotropic effects on the immune system. For example, beige, which is an allele of the lysosomal trafficking regulator (*Lyst*), is demonstrated by its pigment-reducing effect (if not hidden due to an epistatic effect of albino, *c/c*), by a prolonged bleeding time because of a platelet storage pool defect (20 min in homozygous $Lyst^{bg}$ vs 6 min in unaffected wild-type or heterozygous controls), and by abnormal giant lysosomal granules detectable in all tissues with granule-containing cells (histological sections, cytocentrifuge preparations of PBL).

As the determination of a genetic profile is time-consuming and expensive it is only feasible as an initial check. In the case of a variable segregating background, genetic profiling is pointless; the typing results may only assist in determining the degree of heterogeneity, but may provide hints about modifying genes if the stock is being inbred and almost homozygous.

PCR protocols used to demonstrate specific mutant genes are provided for the respective marker in MGD (http://informatics.jax.org; check: Genes, markers and phenotypes, see RFLP/PCR polymorphism) and for induced mutations (maintained at the Jackson Laboratory) that can be distinguished from normal wild-type mice on the World Wide Web (http://www.jax.org/resources/documents/imr/protocols/index.html) or can be obtained through an e-mail inquiry to micetech@aretha.jax.org. Moreover, information on RFLP polymorphisms as determined by a Southern blot (Sambrook *et al.*, 1989) using a specific probe is also provided in MGD, if applicable and available.

Simple measures are needed to distinguish between those strains that are co-maintained and those that clearly identify an outcrossing event. A critical subset of the markers (i.e. the least amount of differentiating marker for a given strain panel) used to authenticate the strains maintained will provide reasonable information about the genetic quality of a strain. Unfortunately, with each strain added to a unit the number of markers in the critical subset increases. Critical subsets need to be verified at regular intervals (every 3–6 months). The intervals and the number of animals to be tested depends on the number of strains co-maintained and the size of each colony.

One of the most powerful pieces of information about an inbred strain is the demonstration of isohistogeneity. This is best demonstrated through skin grafting, which is simple to perform, although time-consuming because of an observation period of about 100 days (Box 9). In specific immunodeficient mutants (e.g. $Hfh11^{nu}$, $Prkdc^{scid}$, $Rag1^{tm1}$, $Rag2^{tm1}$) a direct demonstration of isohistogeneity is impossible as these animals are

incapable of mounting an allorecognition response. This can be circumvented by transferring grafts from these immunodeficient animals to their syngeneic background strains.

Box 9 Orthotopic tail skin grafting

Animals aged 6–9 weeks serve best as recipients for orthotopic tail skin grafting. The animals to be grafted are anaesthetized with a volatile or injectable anaesthetic (ether inhalation or ketamine hydrochloride supplemented with xylazine). The tail is scrubbed with antiseptic solution. The animals are placed in ventral recumbency with the tail pointing towards the operator. Thin sheets of skin are sliced with a scalpel (blade No. 11 or 20) towards the tail base. The cut should be as deep as possible but should not sever the dorsal tail artery or vein. Occasional bleeding may be stopped by one or two drops of epinephrine solution (1 : 1000). For a regular reciprocal circle two (several) grafts are taken per animal at the same time, providing the graft bed to receive the grafts from two (several) other animals. Therefore, the method requires meticulous sample preparation. All grafts have to be of the same size (approximately 6 × 2 mm in mice and 8 × 3 mm in rats). The grafts are placed in Petri dishes on saline-drenched filter paper, and the excised graft beds are covered with gauze sponges moistened in saline until grafts are transferred. Then the appropriate grafts are positioned on the prepared beds such that the direction of hair growth is reversed. Excessive fluid and blood is removed by pressing with dental rolls. The grafts are then fixed with liquid surgical dressing and secured by glass tube slipped onto the tail and fixed with a tape. The tube should not exert any pressure on the tail base, as this could lead to a severe oedema due to blocking of the venous drainage.

After recovery from anaesthesia the animals are housed separately on large wooden shavings or on cellulose sheets for the first 2–3 days until the tube is removed. Regular bedding may be scooped into the tube to absorb moisture and scrape off the grafts (at least the proximal ones) when the glass tube is removed.

Grafts are inspected daily for 2 weeks from day 8–10 onwards. Thereafter a graft appraisal once weekly until day 100 post-grafting will suffice. Genetic outcrosses cause the graft to be rejected in an acute fashion and are thus immediately detected.

Technical failures become evident at the first appraisal. Either the grafts are recorded to be dislodged or to be ripped off with the tube. Orthotopic tail skin grafts are sometimes removed by the animal itself during the first week by grooming. This technical failure (of the first set grafts) is evidenced by the presence of an eschar at or before day 7. Technical failures are in the range of 5–10%.

Scoring may vary from laboratory to laboratory, either by fully describing the graft appearance or by defining scores. Skin grafts should be recorded as rejected if and when they are reduced to a scar.

(cont.)

Box 9 (*cont.*)
Other signs, such as a pasty appearance of the graft epidermis or less than 50% of its original surface being intact, may also be taken as an indication of graft rejection. With the latter two types of assessment the median survival times of allogeneic grafts will be shorter by a few days. Specific scores separately defining the graft by size and outward appearance will ease the appraisal of skin grafts in a large number of animals (Hedrich, 1990).

If there is any doubt about the success of graft acceptance, regrafting is essential (usually within 2–3 weeks). In the case of true incompatibility (not technical failure), the second set graft will be rejected in a hastened and more pronounced manner. Grafts are considered to have been accepted when the entire graft has healed completely with no clear indication of contraction (< 25% shrinkage). The final assessment after 100 days must take account of previous ratings.

In the case of immunodeficient animals incapable of rejecting an allograft, skin is grafted to the immunocompetent background strain. If the mutation itself is not acting antigenically and no graft is rejected this is proof of isohistogenicity within the strain and with the genetic background.

In order to reduce and simplify routine monitoring procedures, techniques that are fast, reliable and cost-effective are preferred. Random amplification of polymorphic DNA by PCR (RAPD) meets these demands. With this method (Williams *et al.*, 1990) anonymous stretches of genomic DNA are amplified using arbitrarily designed single short primer sequences of about 10 nucleotides. Recombinant inbred strains of mice have been distinguished by using this method (Scott *et al.*, 1992), as have various inbred strains of rats including MHC congenics (D. Wedekind and H.J. Hedrich, unpublished).

Box 10 Differentiation of inbred rat strains by PCR using a random primer (RAPD)

1. Genomic DNA is prepared according to the method described by Miller *et al.* (1989) from ear or from blood, using a DNA extraction kit (Quiagen, Hilden, FRG).
2. RAPD primers with arbitrary nucleotide sequences can be purchased from, e.g., Roth, Karlsruhe, FRG. The 10-mer primers are characterized by their GC content (60%, 70%, 80%).
3. The PCR is carried out in a 25-µl reaction volume containing reaction buffer, dNTPs, one random primer, genomic DNA and DNA polymerase. Amplification is performed on a thermal cycler. The conditions for the PCR must be strictly standardized.
4. The amplified fragments are separated by horizontal gel electrophoreses on a 1–1.4% agarose gel in 1 × TBE (0.1 M Tris/borate, 2 mM EDTA), at constant current (100/80 V, 70 mA) for 5–6 h. The PCR products are visualized with ethidium bromide (Fig. 5).

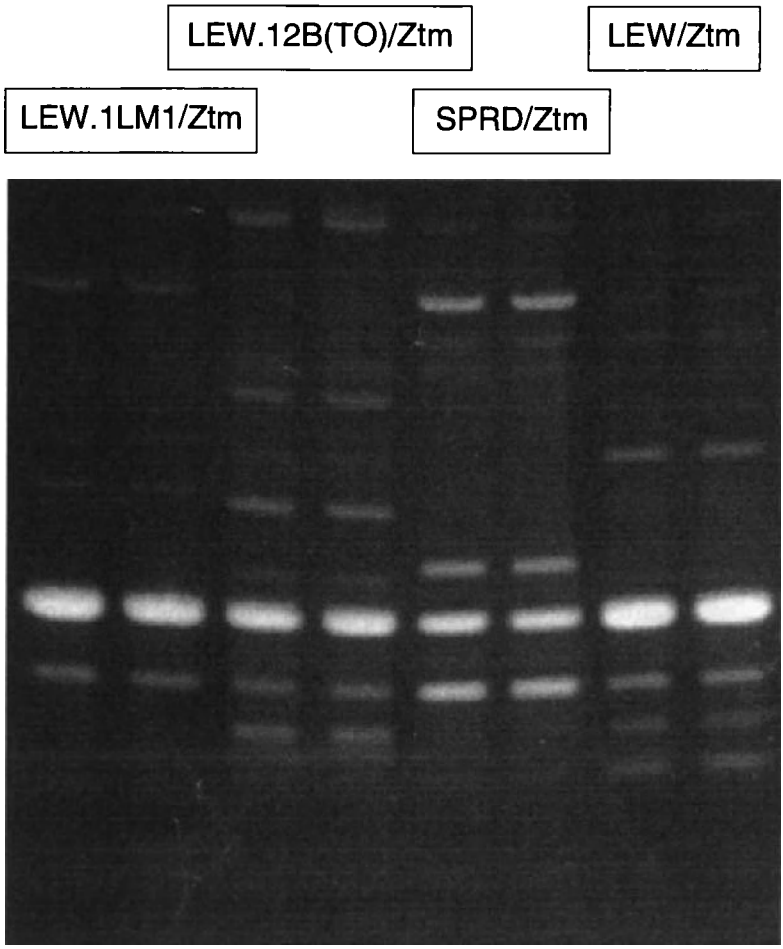


Figure 5. The figure shows the differentiation by RAPD of three congenic inbred rat strains (LEW.1LM1/Ztm, LEW.12B(TO)/Ztm and LEW/Ztm) and one inbred rat strain (SPRD/Ztm).

Another recently developed technique is the demonstration of amplified fragment length polymorphisms (AFLP™, Keygene n.v., Wageningen, Netherlands) (Zabeau and Vos, 1992). The technique is based on the combined use of restriction enzymes and selective PCR primers. Multiple polymorphisms are simultaneously visualized without the need of prior information on genomic sequences. DNA is cleaved into fragments using a set of two restriction enzymes: a rare cutter and a frequent cutter. Adapters are ligated to the ends of the restriction fragments. Adapters that stick to the site of the rare cutter carry a biotin label. Biotin-carrying fragments are isolated by binding to streptavidin beads, resulting in an enormous reduction in the number of fragments. Only fragments containing a rare-cutter end on one side and a frequent-cutter end on the other side, or rare-cutter ends on both sides, will remain in the fragment pool. Subsequently, a further selection will be performed by

PCR using selective primers. The PCR primers overlap the adapters and the restriction sites and are provided with a specific extension at their 3'-ends causing the further selection. The resulting amplicons are separated by PAGE. The AFLP pattern reflects multiple polymorphic markers of presence/absence type, i.e. dominant/recessive markers (Otsen, 1995).

◆◆◆◆◆◆ CRYOPRESERVATION AND REVITALIZATION OF LINES

The high costs of animal care and maintenance often makes it difficult (for a researcher) to maintain strains that are no longer actively used. Furthermore, many individual research colonies are microbiologically contaminated, so that virus-free facilities are reluctant to import mice from unknown sources. Therefore, the freezing of preimplantation embryos is considered to be the proper means to cope with the multiplicity of strains of mice and rats presently available, to serve as a safeguard against loss, to allow for eradication of infections if the embryo transfer is performed under aseptic conditions onto barrier maintained surrogate dams, and to reduce the costs for valuable strains presently not used. Despite certain improvements, the freezing of murine embryos is a time-consuming and cost-effective task. While outbred stocks and hybrids in general respond to superovulation by gonadotrophins (see protocols in Box 11) with a high ovulation rate, inbred strains show a rather variable response. In addition revitalization results also vary substantially on a strain by strain basis, and strongly depend on the skill of the personnel. Therefore, it has not been possible to preserve as many strains recently developed by molecular genetic methods as necessary. Freezing of sperm, if sufficiently efficient, could assist in this task. As with sperm freezing the protocols for *in vitro* fertilization (IVF) (see Box 4) also need to be improved.

The original technique of embryo freezing as described by Whittingham *et al.* (1972) and Wilmut (1972) requires a controlled slow freezing and slow thawing procedure with dimethylsulphoxide (DMSO) or glycerol as the cryoprotectant. Since this first description of successful freezing of eight-cell mouse embryos, various modifications in the use of cryoprotectants, freezing methods and freezing of other developmental stages have been reported (for an overview see Hedrich and Reetz (1990)).

Freezing of embryos at a slow speed (0.3–0.8°C min^{-1}) to –80°C permits the embryos to undergo progressive dehydration, thus preventing intracellular ice-crystal formation. Thawing has then to be slow (about 8°C min^{-1}) in order to allow the blastomeres to rehydrate without deleterious side-effects. This is the method established and used at The Jackson Laboratory, Bar Harbor, ME, USA.

The procedure of freezing embryos at a low rate (0.4°C) to a subzero temperature of only –30°C to –40°C with subsequent immersion in liquid nitrogen requires thawing at about 300–500°C min^{-1}. The latter method is

less time-consuming, less expensive, and more practical types of freezer are available. However, embryos frozen by a 'fast' technique are thought to be in a metastable state and very slight alterations during warming and cryoprotectant removal might damage the embryos seriously (Leibo, 1981). The success of revitalization not only depends on strain/species and on the freeze–thaw technique (Rall *et al.*, 1980; Rall, 1981), but also on the embryo transfer and the skill of the practitioner. The two-step freezing and vitrification procedures established in the authors' laboratories, which have been shown to give reasonable results with two-cell embryos from mice and rats, are given in Boxes 12 and 13.

The selection of embryo donors depends on the type of strain to be cryopreserved. In the case of an outbred stock the group of revitalized breeding pairs required to build up a new colony should be genetically equivalent to their colony of origin, i.e. the genotype distribution within both populations should be equivalent. Furthermore, mating of close relatives must be avoided to ensure that the coefficient of inbreeding is kept at a low level. A random sample of breeders collected from the source colony may fulfill this requirement and serve as donor parents. In practice this can be realized if only embryos with different ancestors are frozen in one single cryotube (subsequently termed 'embryo batch') and if a number n of independent batches is used to rederive a new colony. In dealing with inbred strains it is to be differentiated whether a foundation colony is to be restocked or whether the frozen embryos should serve in future as breeders of an expansion colony. The former have to be derived from a single pedigreed breeding pair, preferably originating from the foundation colony. Restocking of an expansion colony also calls for pedigreed embryos, but these can be derived from different donors as strict brother × sister mating is not mandatory. In the case of congenic (CR) and mutant, as well as most transgenic or knock-out lines, it is the primary objective to maintain the differential or mutant/mutated gene. For this purpose a pool of embryos (descending from different donors) may suffice. After rederivation, however, one to four back-cross cycles to the background strain are required.

It has been shown in mice as well as rats that all preimplantational stages can be revitalized successfully upon freeze–thaw procedures. For long-term storage eight-cell stages have been recommended, while two-cell stages were considered to be less suitable. Results obtained in the authors' laboratories (see also Mendes da Cruz, 1991) show that frozen–thawed two-cell embryos can be revitalized at a comparable rate. The two-cell embryos are transferred into the oviducts of day-1 surrogate dams, eight-cell embryos may be transferred into the oviducts of day 1–2 surrogate dams, or into the uterus after a 24 h culture period.

One embryo batch (inbred strain) derived from a single pedigree donor pair may be regarded as a prospective breeding nucleus, if one fertile breeding pair is obtained upon revitalization. Assuming an average revitalization rate of 20% (fertile breeders), one embryo batch should contain a minimum number of 10 embryos to obtain at least one breeding pair with a 50% chance of revitalization (Table 7). According to these figures, 100 cryopreserved embryos is to be regarded as the lower

Table 7. Minimum number of embryo batches needed to obtain at least one B × S breeding pair as progenitors for a new nucleus*

No. of embryos per batch	Expected no. of weanlings after revitalization	No. of batches needed (probability for ≤1 B × S pair)	
		99.0%	99.9%
10	2	6.7	10.0
15	3	3.5	5.0
20	4	2.2	3.3

* Based on an average revitalization rate (weaned and reproductive for inbred strains of about 20%).

limit to provide a safe backup of a strain. Nevertheless, a safe backup of a strain is affected not only by the size and the number of embryo batches frozen, but also by the revitalization rate significantly differing between strains as well as according to the skill of the staff in embryo transfer techniques. For routine embryo banking, therefore, ≥20 embryo batches per strain, each containing 10–20 embryos, will be sufficient to guarantee a safe backup.

Effective superovulation protocols thus are crucial. The average rate of embryo batches per hormone-treated female (≥10 two-cell embryos per batch per female) amounts to about 30% (range 10–43%). For eight-cell embryos this rate drops to 20–25% (H. J. Hedrich and I. Reetz, unpublished data). If a strain is refractory to superovulation (as it is for certain inbred strains and even non-inbred transgenic stocks), the embryos are obtained from normal mated donor females.

Embryos forming an embryo batch are frozen together in a freezing container. Various types of container are in use, such as 2-ml polycarbonate tubes with screw caps, glass or polypropylene ampoules, and plastic straws. When sterilizing heat-labile embryo containers with ethylene oxide, one has to consider the cytotoxic effect of the absorbed retained gas. Containers sterilized by this method must not be used until a sufficient post-sterilization aeration (approximately 3 weeks) has been completed (Schiewe *et al.*, 1985). There are, however, straws available that withstand heat sterilization. To avoid mixing up embryo batches, each container must be permanently marked with the strain name and strain code. This is facilitated by using a hand-driven printing device. In addition, each freeze run must be monitored by means of a temperature recording, and its reliability should be monitored by using a vitality test of an additional embryo batch of an F1 hybrid or outbred stock highly responsive to superovulation. After the freeze run the cryocontainers should be properly placed at defined locations/compartments within the embryo repository in a properly controlled liquid nitrogen refrigerator. It is important to keep full records, which should include the conditions of the freeze run (type, cryoprotectant used, etc), a strain description, and an identification and storage location. The physical conditions of the freezing procedure, including results of viability tests obtained from the particular control batch, are required as documentation of a correct freezing technique and to provide further information on the thawing procedure to be applied. For each stock

in the repository a description of the strain or mutant, with particular information about phenotype, reproductive performance and strain history, should be kept on a file. Information concerning identification requires complete pedigree information, such as parentage, genotype, generation, the code number of the embryo batch, the number and developmental stage of embryos frozen, and a precise storage location.

The funds needed to run an embryo bank have to cover the personnel expenditure (at least one scientist and one technician), the cost of basic equipment (investment with 10 years amortization) and the running costs. The estimated cost per year is about US$ 90 000–100 000. The cost of maintaining a breeding nucleus of one strain under specified pathogen-free conditions is about US$ 4500–5500. Thus cost equivalence is achieved if approximately 20 strains have been deposited in the repository and are no longer maintained as vital breeding nuclei in the animal quarter.

Sperm freezing, although not well established, could assist in all cases where animal-holding space is limited and strains do not respond well to superovulation. This primarily applies to colonies of mice bearing mutations or transgenes. Recent attempts at sperm freezing (Table 8) associated with IVF are promising.

Box 11

Superovulation protocol for the mouse:

The protocol given below is that described by Whittingham (1971).

- *Animals:* females aged 6 weeks to 4 months.

1. *Day –2, 16.00 h:* inject 5–10 iu pregnant mare's serum gonadotrophin (PMSG).
2. *Day ±0, 16.00 h:* inject 5 iu human chorionic gonadotrophin (hCG), mate to males.
3. *Day +1, morning:* check for the presence of a vaginal plug.
4. *Days +1 to +4:* collect preimplantatory embryos.

Superovulation protocol for the rat:

The protocol given below is a modification of that described by Rouleau *et al.* (1993).

- *Animals:* adult females, at least 59 days old.

1. *Day –4, 08.00 h:* inject 40–60 µg luteinizing hormone releasing hormone (LHRH).
2. *Day –3, 17.00 h:* load the osmotic minipump with follicle stimulating hormone (FSH), so that 6.8 mg FSH is delivered daily. (The pump is kept in sterile saline at room temperature until use in order to reach the nominal steady state value.)
3. *Day –2, 08.00 h:* implant the osmotic pump.
4. *Day ±0, 16.00 h:* inject 30 iu human chorionic gonadotrophin (hCG), mate to males.
5. *Day +1, 08.00 h:* plug control and vaginal cytology.
6. *Days +1 to +5:* collect preimplantatatory embryos.

Table 8. Cryopreservation of mouse spermatozoa

	Songsasen *et al.* (1997)	Sztein *et al.* (1997)	Dorsch and Hedrich (unpublished)
Animals	F1 hybrid	F1 hybrid	Inbred strains
Freezing medium	0.3 M raffinose, 0.2 M glycerol, egg yolk	18% raffinose, 3% skim milk (Nakagata and Takeshima, 1992)	18% raffinose, 3% skim milk (Nakagata and Takeshima, 1992)
Freezing procedure	At slow speed to –20ºC, then transfer to LN_2	Transfer to –120ºC, then transfer to LN_2	5 min on dry ice, then transfer to LN_2
Thawing procedure	Fast	Fast	Fast
Dilution of freezing medium	One step (1:10), centrifugation	One step (1:10), centrifugation	One step (1:10)
Sperm motility after thawing	Approx. 30%	Approx. 80–85%	Approx. 50%
In vitro *fertilization:*			
Fertilization ability	Approx. 30%	Approx. 85–90%	Approx. 80–85%
Developmental ability	>30%		25–30%
Implantation/offspring	30–35%	37%	Approx. 10%

Box 12 Two-step freezing of embryos

The procedure for freezing embryos at a low rate to a temperature between –30°C and –40°C following the conventional protocol with subsequent immersion in liquid nitrogen has certain advantages. For instance it is less time consuming, less expensive, and more practical types of apparatus are available or may be designed.

Consistent results have been obtained in the authors' laboratory by applying a two-step freezing technique. For freezing, plastic straws are used instead of other containers because they can be better marked and almost no embryos get lost during manipulation. The straws are loaded, with the embryos being placed in the centre of the medium column. In a programmable automatic ethanol cooling bath the embryos are equilibrated at 0°C in freezing medium with 2.0 M propanediol as the cryoprotectant for 5 min, and then cooled to –6°C at a rate of 1°C min^{-1}, seeded (induction of extracellular ice crystal formation by touching the straw at the air–medium interface with a metal rod precooled in liquid nitrogen) and then slowly cooled to –32°C at a rate of 0.4°C min^{-1}, held for about 5 min at –32°C, and transferred directly to the liquid nitrogen refrigerator. The straws are always handled horizontally to keep the embryos in position, until seeding is finished.

Thawing at a rate of about 300°C min^{-1} is achieved by warming the straws at room temperature for about 40 s (Renard and Babinet, 1984; Mendes da Cruz, 1991).

Box 13 Vitrification of embryos (quick freeze/fast thaw procedure)

There are a number of reports that mouse and rat embryos survive freezing after rapid cooling by directly plunging into liquid nitrogen. This quick freeze/fast thaw procedure requires the use of a highly concentrated aqueous solution of cryoprotectants. At sufficiently low temperatures, these solutions become so viscous that they turn into an amorphous state without any formation of ice. This process has been termed 'vitrification'. Most groups use glycerol (3.0–4.0 M) as a permeable and sucrose (0.25–1.0 M) as a non-permeable cryoprotectant. Before freezing the embryos must be dehydrated. At temperatures below 4°C embryos can tolerate exposure to a concentrated solution of cryoprotectants and the associated osmotic dehydration. The following method, based on the one reported by Rall and Fahy (1985), has been shown to give reasonable results in mice and rats.

The vitrification solution (VS1, pH 8.0) is composed of a mixture of 20.5% DMSO (w/v), 15.5% acetamide (w/v), 10.0% propanediol (w/v), 6.0% polyethylene glycol (w/v) in PB1 (mice), or TCM 199 supplemented with 20% heat-inactivated rat serum (sTCM, rats). Embryos are equilibrated at about 0°C (on ice) in four steps at different

(*cont.*)

Box 13 (*cont.*)
concentrations of VS1. Each equilibration step with 12.5%, 25%, 50% and 100% VS1 is exactly timed for 10 min. Then the embryos are transferred to a straw (e.g. Minitüb) containing VS1. Immediately thereafter the straw is sealed and exposed to –196°C (liquid nitrogen), care being taken to keep the straw in a horizontal position during all manipulations.

Although this method does not require an elaborate biofreezer and appears to be rather simple, it has not yet replaced the more conventional techniques. The conditions for pre-dehydration and cryoprotectant removal require further optimizing. Post-thaw survival is variable for the different developmental stages of the embryos, and may depend on the type, concentration, temperature and equilibration time of the cryoprotective solution used for vitrification. In contrast to these requirements, dehydration and removal of cryoprotectant is less critical for the survival of embryos frozen by the two-step method as used routinely in the authors' laboratory (Box 12).

The requirements for thawing embryos are defined by the freezing procedure and the cryoprotectants used. The manipulation depends on the cryocontainer used. In tubes and ampoules a few embryos regularly get lost because they stick to the wall, whereas with straws all embryos are usually recovered.

The straw containing the embryo batch to be revitalized is removed from the liquid nitrogen container. Both tips holding the sealing bulbs are cut off and the straw is attached to a syringe filled with air. When all ice crystals have disappeared (after about 40–45 s), the cryoprotectant solution containing the embryos is gently flushed into an equal amount of PB1 (mice) or sTCM (rats) to reduce the concentration of the cryoprotectant by 50%. After two further stepwise elutions (25%, 12.5%), always with a 10-min equilibration, the embryos are put through five washes in sterile medium, and held for up to 30 min. This permits the embryos to recover from osmotic distress. Embryos that appear to be morphologically unimpaired by microscopic inspection are selected for immediate transfer to pseudopregnant recipients.

◆◆◆◆◆◆ MANAGEMENT OF INFECTED COLONIES

'Natural Infections'

Quarantine/infections

Animals with an unknown microbiological status have to be kept in isolation until the examination is finished. This mostly concerns animals received from other institutions. The state of isolation should be the same

as for infected animals. Because, in general, a broad range of gene-manipulated stocks, which are potentially infected with different pathogens, has to be accepted from outside further isolation from each other should be accomplished. Although ventilated cabinets, IVCs or isolators may be used, the need for rederivation is obvious. The same is true for animals with an unwanted microbiological status which are a risk to the whole facility.

Rederivation

Hysterectomy

As shown for most infections, the vertical transmission of viral, bacterial and parasitic pathogens can be avoided by this procedure. The protocol originally recommended by Trexler (1983) is depicted in Box 14.

The most difficult part of this procedure is to achieve timed pregnancy, especially in poorly breeding strains. This method is recommended if embryo transfer cannot be performed due to lack of equipment and trained personnel, or eventually in the case of a donor strain that is refractory to superovulation. Hysterectomy has the additional risk of intrauterine vertical transmission of micro-organisms, which may be higher in immunodeficient than in immunocompetent animals.

Box 14 Hysterectomy

1. Mate foster mother (outbred or hybrid strain) in the clean area overnight; check for vaginal plug.
2. 24–48 h later, mate animals of the microbiologically contaminated strain; check for vaginal plug.
3. Install the dip tank filled with low-odour disinfectant before the expected date of birth of the foster mother.
4. Shortly before delivery, kill the pregnant dam of the strain to be rederived by cervical dislocation; carry out hysterectomy under aseptic conditions.
5. Transfer the uterus to the clean side through the disinfectant (38°C).
6. Wash the uterus intensively in physiological saline, and develop the pups.
7. As an extra safety precaution, the pups may be dipped again in disinfectant and washed again in physiological saline.
8. After gentle massage with a swab to induce spontaneous breathing and after warming up, transfer the pups to the nest of the foster mother after disposing of her own offspring.
9. If coat-colour discrimination is possible, one or two of the foster mother's pups may be retained to assist in the induction of lactation.

Embryo transfer

Embryo transfer has been shown to interrupt most vertically transmitted infections of viral, bacterial or parasitic origin, with the exception of germ line transmitted retroviral infections. The integrity of the zona pellucida is of decisive importance, as shown for MHV infection (Reetz *et al.*, 1988). The hygiene status of the foster mother should be of the highest level, especially when a new breeding unit is to be established. For routine procedures, two-cell stage embryos may be the most suitable, because fertilization is no longer in question and a higher number of embryos can be collected than at later stages. The animals are mated overnight without or after previous superovulation (for details see Box 11) (Reetz *et al.*, 1988; Hogan *et al.*, 1994; Schenkel, 1995). The latter method allows synchronization of mating, and generally induces production of higher numbers of embryos than by normal mating. The embryos are flushed from the oviducts of plug-positive mice on day 1.5 and washed at least four times in large volumes of media (approximately 2 ml) at a different location before being transferred to the clean area where implantation into the oviducts of pseudopregnant recipients is performed by other staff. Pseudopregnancy is induced by mating with either surgically sterilized or genetically sterile males (see Box 5).

Embryo transfer offers specific advantages over hysterectomy: it avoids the risk of intrauterine vertical transmission of infections; it allows easier timing, especially in the case of superovulation; and it allows cryopreservation of surplus embryos. For special applications we have developed a method for flushing embryos *in vivo*, allowing the use of embryo donors for successive reflushing or 'normal' breeding. This procedure requires extreme skill and cannot be recommended for routine manipulation.

Furthermore, new strains shipped as cryopreserved embryos can be transferred to surrogate dams with the specific SPF status, avoiding time-consuming quarantine and rederivation procedures. Moreover, accidental infection during shipment can be avoided.

Therapeutic treatment

In general, the administration of drugs influences the outcome of animal experiments and cannot be considered as a substitute for improving hygiene standards. The success of treatment depends on several criteria: a correct diagnosis, and consideration of species-specific toxicity, adverse reactions, optimal dosage and regimen of application, accompanying hygiene procedures, etc. Unfortunately, recommended dosages often refer to man or larger animals. For extrapolation to small rodents allometric parameters should be used, which increase the body weight ratio by a factor of approximately 6 and 12 for rat and mouse, respectively, in comparison to man (for a review see Morris, 1995). In addition, the half-life of drugs is, in general, reduced, thus requiring more frequent administration for an effective level of drug to be maintained.

In general, the treatment of parasitic invasions has to be accompanied by hygiene procedures (e.g. chemical and physical disinfection, change of cage). Some commonly used antiparasitic drugs are listed in Table 9. Chemotherapeutic and antibiotic treatment of infections may induce resistance, overgrowth of other bacterial species (Hansen, 1995), or adverse reactions by altering the gut flora (Morris, 1995). Commonly used antibacterial treatments are listed in Table 10 (see also Hawk and Leary, 1995). It should be stressed, however, that the use of therapeutic drugs will reduce, but only occasionally eliminate, parasites or micro-organisms.

Preventive therapeutic treatment may be of help in providing a better chance of transferring quarantined and accidently infected animals. In immunocompromised animals drugs are used to suppress opportunistic infections, especially those of human origin.

Experimental Infections

General precautions

The safe operation of an animal laboratory is one of the main management responsibilities. Housing infected animals requires precautions to prevent transmission of micro-organisms between animal populations and, in the case of zoonotic agents, to humans. The zoonotic risk arising from naturally infected rodents is low because most rodent pathogens do not infect man. Only a few agents like LCMV, Hantaviruses or *Streptobacillus moniliformis* have the potential to cause severe infections in humans and might be prevalent in colonies of laboratory rodents. Severe disease outbreaks in humans associated with infected colonies of laboratory rodents have been reported (Bowen *et al.*, 1975; Kawamata *et al.*, 1987), and therefore safety programmes are necessary to prevent laboratory-associated infections and infections transmitted by laboratory animals.

Experimental infections are more likely to pose a risk for humans. A broad spectrum of infectious agents can be introduced accidentally with patient specimens, and many laboratory animals are still used for infection experiments. In general, health precautions are very similar for clinical or research laboratories and for animal facilities. In many cases, however, an increased risk may arise from experimentally infected animals due to bite wound infections or when pathogens are transmissible by dust or by aerosols.

A number of recommendations exist from federal authorities for microbiological laboratories, aimed at the prevention of infection of laboratory personnel. Many programmes were developed in response to evaluations of laboratory accidents. Most laboratories have written control plans that have been designed to minimize or eliminate risks for employees.

Reduction of the risk of disease transmission can be achieved by very general procedures which are common practice in most well-run facilities housing animals behind barriers. Only major points can be discussed

Table 9. Treatment of common parasites (in combination with hygienic measures)

Generic name	Trade name	Application	Dose	Reference
Ectoparasites				
See table in Weisbroth (1982)				
Ivermectin*	Ivomec (Merck & Co)	Topical spray	0.2–10 mg ml^{-1}	Hirsjärvi and Phyälä (1995)
Endoparasites†				
Piperazine citrate		Drinking water (for 12 weeks, every 2nd week)	0.2%	Maess and Kunstyr (1981)
Fenbendazole‡	Panacur (Hoechst)	Diet‡ several months	100–150 ppm in diet or 25–50 mg kg^{-1} body weight	Strasser and Tiefenbach (1977)
Ivermectin*	Ivomec	Topical spray 2 ml/cage	1 mg ml^{-1}, 2 ml/cage, once weekly for 3 weeks	Le Blanc *et al.* (1993)
		Drinking water	2.9–4.0 mg kg^{-1} for 4 days, 3-day pause, 5 cycles	Klement *et al.* (1996)
Ivermectin–piperazine (combined)		Drinking water	7000 ppm, 2.1 mg ml^{-1}, alternately every 2 weeks for several months	Lipman *et al.* (1994)

For disinfection especially in the case of parasite eggs and oocysts, a chlorocresol formulation has proven valuable (Neopredisan, Menno-Chemie Vertrieb GmbH, 22850 Norderstedt, Germany). For additional drug dosages, see Hawk and Leary (1995).

* Toxic in young mice (Scopets *et al.*, 1996).

† Especially *Syphacia obvelata* and *Aspicularis tetraptera*.

‡ Diet can be autoclaved without substantial loss of efficacy.

Table 10. Selected antibiotic therapies for small rodents

Disease/species	Anti-infective	Application in drinking water	Dose	Reference
Pasteurellosis/mouse	Enrofloxacin (Baytril)	For at least 30 days	25.5–85 mg kg^{-1}	Goelz *et al.* (1996)
Mycoplasma/rat	Oxytetracycline	For at least 5 days*	3–5 mg ml^{-1}	Harkness and Wagner (1983)
	Tylosin	For 21 days	5 g l^{-1}	Carter *et al.* (1987)
Hepatitis–typhlitis/ mouse (*Helicobacter hepaticus*†)	Amoxicillin‡	For 4 weeks – young mice	50 mg kg^{-1}	Russell *et al.* (1995)
	Amoxicillin‡ Metronidozole Bismuth	For 2 weeks	200 mg l^{-1} 138 mg l^{-1} 37 mg l^{-1}	Foltz *et al.* (1996)
Pneumocystosis/mouse, rat	Sulphamethoxazole–trimethoprim (Borgal; Cotrim K, Ratiopharm)	For 3 weeks	200 mg–1 g l^{-1} 40–200 mg l^{-1} (5 ml l^{-1})	H. J. Hedrich (unpublished) Rülicke (personal communication)
Tyzzer's disease (*Clostridium piliforme*)/ mouse, rat, rabbit	No antibiotic therapy recommended; rederivation Special disinfectant required§			

For additional drug dosages see Hawk and Leary (1995).

* Drinking water should not be acidified; addition of 1.35 g l^{-1} potassium sorbat prevents growth of yeast.

† Especially immunodeficient mice.

‡ Toxic for hamsters and guinea-pigs.

§ Chlorocresol (Neopredisan)

here; more details on general laboratory safety are given in many textbooks on clinical microbiology (Gröschel and Strain, 1991; Burkhart, 1992) and in general recommendations for housing of laboratory animals (CCAC, 1980; Bruhin, 1989; Kunstyr, 1988b; BG Chemie, 1990; National Research Council, 1996).

Education is an important part of an effective safety programme. All safety instructions should be in written form and must be readily available at all times.

The first point must be adherence to safety procedures and proper behaviour, such as use of personal protective clothing. Prohibition of eating, drinking, smoking, handling of contact lenses and the application of cosmetics in the laboratory are other basic rules, as is the separation of food storage refrigerators from laboratory refrigerators. The most likely route of infection is direct contact with contaminated animals or materials. Micro-organisms do not usually penetrate intact skin. The risk of infection can therefore be reduced by repeated hand decontamination and by decontamination of surfaces or contaminated instruments.

Working with infectious agents should not be permitted in cases of burned, scratched or dermatitic skin. Needles and other sharp instruments should be used only when necessary, and handling of infected animals should be allowed only by experienced and skilled personnel, to prevent bite wounds. Working in safety cabinets helps to avoid inhalation of infectious aerosols and airborne particles which are easily generated in cages when animals scratch or play. Other procedures that might bring organisms directly onto mucous membranes are mouth pipetting and hand–mucosa contact. Both must be strictly forbidden.

Most animal facilities are constructed in such a way that proper cleaning and disinfection can be performed easily, which helps to control infectious animal experiments. In contrast to clinical laboratories, there is usually no wooden furniture and sufficient space is provided to allow access for cleaning and disinfecting whole rooms or areas.

Microisolator cages are often used in animal facilities for transportation within the facility in order to avoid exposure of humans to allergens. Such cages, too, help to reduce the risk of spreading micro-organisms during transportation.

In most animal facilities containment equipment (microisolator cages, isolators) is used if immunosuppressed animals have to be protected from the environment or if infected animals might be a hazard to humans or other animals. Experiments with infectious agents will usually be conducted in separate areas that fulfil all safety requirements such as ventilation (negative pressure in laboratories to prevent air flow into non-laboratory areas) or, better, in isolators which represent the most stringent containment system. For safety reasons, containment is generally necessary if animals are artificially infected with pathogenic micro-organisms. Various systems can be used, depending on the properties of the agents (e.g. pathogenicity, environmental stability, spreading characteristics). In the case of low pathogenic organisms, microisolator cages might be sufficient. The risk of infection during handling is reduced if all work with

open cages is conducted in changing cabinets or in laminar flow benches. Individually ventilated cages operating with a negative pressure are more suitable than microisolators to prevent spread of micro-organisms if they are handled properly. The highest level of safety can be achieved by using a negative-pressure isolator. If handling through thick gloves is not possible, handling of animals can be performed in safety cabinets that can be docked directly to the isolator.

An important part of safety programmes in laboratories, and especially in laboratory animal facilities, is waste management. In contrast to radioactive or chemical waste, infectious waste cannot be identified objectively. In many cases judgement of whether waste from animals that are not experimentally infected is infectious or not is dependent on the person in charge. There is, however, no doubt if animals have been infected experimentally. In such cases the presence of a pathogen allows evaluation of the risk, which depends on the virulence and the expected concentration of an agent together with the resistance of a host and the dose that is necessary to cause an infection. The risk of pathogen transmission is increased by injuries with sharp items such as needles, scalpels or broken contaminated glass. Segregation of such sharp items and storage in separate containers is necessary to keep the risk of infection to a minimum.

Infectious waste from animal houses (bedding material, animal carcasses) can be submitted to chemical or thermal disinfection, but incineration and steam sterilization are the most common treatment methods. Incineration has the advantage of greatly reducing the volume of treated materials. The usually low content of plastic material in waste from animal housing and the high percentage of bedding material (e.g. wood shavings) resulting in a high-energy yield make incineration the method of choice.

Safety levels

Classification of micro-organisms

Four different safety levels have been established (Centers for Disease Control/National Institute of Health, 1993), which consist of combinations of laboratory practices and techniques, safety equipment and recommendations for operation of laboratory facilities. The classification of an organism or parts of it (DNA, toxin) is based on various factors such as the host spectrum, virulence for healthy humans and animals, minimal infectious dose, mode of transmission, epidemiological situation (prevalence in a given population), availability of antibiotics, vaccines or other treatments, and tenacity (Table 11). The recommended levels represent those conditions under which the organism can ordinarily be safely handled. Sometimes, more stringent practices may be necessary when specific information is available to suggest that virulence, pathogenicity, vaccine and treatment availability or other factors are altered. For example, hantaviruses are typical BL-3 pathogens. Many researchers

Table 11. Examples of micro-organisms belonging to different biosafety levels

BL1	BL2	BL3	BL4
Viruses			
Phages	Hepatitis B virus	Eastern equine encephalitis virus	Marburg virus
Tobacco mosaic virus	Herpes simplex virus	Herpes B virus	Ebola virus
	SV 40	Human immunodeficiency virus	Smallpox
	Parvovirus B19	Yellow fever virus	Lassa fever virus
	Vacciniavirus		Foot and mouth disease
	Rabies virus		African swine fever
	Mouse adeno virus		
Bacteria			
Arthrobacter sp.	*Borellia burgdorferi*	*Bacillus anthracis*	None
Bacillus subtilis	*Clostridium tetani*	*Brucella melitensis*	
Erwinia sp.	*Escherichia coli*	*Chlamydia psittaci*	
Lactobacillus acidophilus	*Enterococcus faecium*	*Coxiella burneti*	
Micrococcus luteus	*Listeria monocytogenes*	*Mycobacterium tuberculosis*	
Oligella urethralis	*Mycoplasma pneumoniae*	*Mycobacterium leprae*	
Pseudomonas fluorescens	*Pasteurella multocida*	*Pseudomonas mallei*	
Streptomyces sp.	*Salmonella enteritidis*	*Rickettsia prowazekii*	
	Staphylococcus aureus	*Yersinia pestis*	
	Vibrio cholerae		
Fungi			
Aspergillus niger	*Aspergillus fumigatus*	*Blastomyces dermatitidis*	None
Alternaria alternata	*Candida albicans*	*Coccidioides immitis*	
Malassezia furfur	*Cryptococcus neoformans*	*Histoplasma capsulatum*	
Saccharomyces cerevisiae	*Microsporum canis*	*Paracoccidioides brasiliensis*	
Rhizopus oryzae	*Trichophyton mentagrophytes*		
Parasites			
Apathogenic trypanosomas	*Leishmania donovani**	*Leishmania donovani*†	None
	Eimeria sp.		
	Trichinella spiralis		

* Work without vector.

† Work with vector.

consider hantaviruses BL-4 agents when inoculated into laboratory animals, especially into rats, since there is clear evidence of aerosol transmission from infected animals. The safety instructions are applicable for working with organs, tissues or cells that contain or may shed microorganisms. For example, many cell lines have been immortalized by SV40 virus and may shed this pathogen. Such cells, like the virus, should be handled by using biosafety level 2 practices.

The biosafety level assigned to an agent is based on activities typically associated with the manipulation of quantities and concentrations of infectious agents required to accomplish its identification. If activities require larger volumes or higher concentrations or manipulations which are likely to produce aerosols, additional personnel precautions and increased levels of containment are indicated. Details on all aspects of biosafety have been published by the Centers for Disease Control/National Institute of Health (1993) and BG Chemie (1990, 1991a,b,c, 1992).

- *Biological safety level 1 (BL-1)* applies to the use of characterized microorganisms not known to cause disease in healthy human adults. BL-1 organisms are, for example, attenuated viruses that are used for the production of life vaccines (e.g. polio vaccine), or viruses that are apathogenic for man and animals. Life vaccines for animals containing viruses that may be pathogenic for humans may keep their pathogenic properties for humans. Therefore, a Newcastle disease vaccine, although apathogenic for poultry, is classified as level 2. Other level 1 pathogens are many plant viruses (e.g. tobacco mosaic virus) and viruses of bacteria and fungi (phages). All bacteria that do not multiply in warm blooded organisms, saprophytes and bacteria that have been used for the production of foodstuffs (e.g. lactobacilli) or the preservation of vegetables are also classified as class 1. Fungi that do not infect healthy humans (even if they have the potential to infect immunocompromised hosts) are classified as level 1 (e.g. *Saccharomyces cerevisiae, Malassezia furfur, Aspergillus niger*).
- *Biological safety level 2 (BL-2)* is used for work involving agents that represent a moderate hazard for personnel and the environment, for farm or wild-living animals, or for plants. This level is applicable to clinical, diagnostic, teaching and other facilities in which work is done with a broad spectrum of agents that are present in a population and are associated with human or animal disease of varying severity. Most vertebrate viruses and a broad spectrum of bacteria (e.g. *E. coli, Staph. aureus, Clostridium tetani, Vibrio cholerae*) are classified as BL-2 organisms. Fungi that may infect healthy humans or animals (e.g. *Candida albicans, Trichophyton mentagrophytes, Microsporum canis, Aspergillus fumigatus*) and for which efficient drugs are available are also classified as biosafety level 2. Classification of parasites is in many cases dependent on additional factors that may have impact on the risk. Most pathogenic parasites (protozoans, trematodes, nematodes, mites and insects, including lice and fleas) are classified as BL-2. For some protozoal parasites, the primary laboratory hazard arises from transmission by arthropod vectors. For such parasites (e.g. *Trypanosoma cruzi, Leishmania donovani, Plasmodium falciparum*) working is only classified as BL-2 if vectors necessary for transmission are not used. For other

parasites a different risk may arise from larval stages or from adult parasites. For several trematodes the most serious risk arises from larval stages (e.g. *Fasciola hepatica, Dicrocoelium* sp., *Paragonimus* sp., *Opisthorchis* sp., *Schistosoma* sp.). Working with larvae, therefore, requires BL-2 practices, whereas working with adult worms is classified as BL-1. In contrast, working with *Echinococcus* sp. should be conducted under BL-2 conditions only if it is restricted to hydatid cysts, whereas working with adults requires a higher safety level.

- *Biological safety level 3 (BL-3)* is used when working is necessary with indigenous or exotic agents that may cause serious or potentially lethal disease as a result of autoinoculation or ingestion or with a potential for aerosol transmission. Among viruses, yellow fever virus, human immunodeficiency virus (HIV) or herpes virus B are classified as BL-3 pathogens. Only few bacterial species (*Mycobacterium tuberculosis, Yersinia pestis*, Rickettsiae, *Pseudomonas mallei, Coxiella burneti, Brucella melitensis, Chlamydia psittaci*) or fungi (*Blastomyces dermatitidis, Coccidioides immitis, Histoplasma capsulatum*) pose a serious risk to laboratory workers or animals or a moderate risk to the population, and may therefore be classified as BL-3. Few parasites are classified as BL-3 organisms if working includes use of vectors (e.g. *Trypanosoma cruzi*, several *Leishmania* species like *L. donovani, Plasmodium falciparum*). In the case of *Echinococcus* species (e.g. *E. granulosus, E. multilocularis*), working with adult worms may be classified as BL-3, whereas BL-2 is applicable if working is restricted to hydatid cysts.
- *Biological safety level 4 (BL-4)* practices are applicable for work with highly contagious and pathogenic or exotic organisms that may cause lethal infections, for which there is no available vaccine or therapy and which may be transmitted by the aerosol route. Additional agents with a close relationship should also be manipulated at the BL-4 level. Examples are Lassa fever virus, Marburg virus, Ebola virus, or smallpox. Of the animal pathogens, rinderpest, foot and mouth disease and African swine fever should be considered as class 4 pathogens. At present, no bacterial pathogens, fungi or parasites are classified as BL-4.

Safety precautions

- *Biological safety level 1 (BL-1)* requires basic laboratory facilities and the use of standard laboratory practices. No additional safety precautions are necessary if animals are infected with BL-1 pathogens.
- *Biological safety level 2 (BL-2)* requires level 1 practices plus additional measures such as the wearing of laboratory coats and protective gloves. Access is only allowed for persons having specific training in handling pathogenic agents (and technical staff, if necessary); public traffic is not permitted. No protective clothes should be used outside the BL-2 area and all clothes must be autoclaved before washing. Biohazard warning signs must be posted at the entrance door. Food is not allowed to be stored. All persons working in a BL-2 unit should inform supervisors on specific incidents that might influence their resistance to pathogenic micro-organisms (e.g. pregnancy, immunomodulation) as well as on bite or scratch wounds from infected animals.

Persons who are at increased risk of acquiring infection are not allowed in the animal room. All infectious waste must be decontaminated before leaving a BL-2 unit.

Clinical specimens (blood, body fluids, tissues) that may contain micro-organisms pathogenic for humans should be handled using BL-2 practices. Standard precautions include the use of a biological safety cabinet or a biohazard hood when working with any clinical material. These cabinets are the most commonly used primary containment devices in laboratories working with infectious agents. Biological safety cabinets offer the additional advantage of protecting the clinical specimens from extraneous airborne contamination.

- *Biological safety level 3 (BL-3)* requires level 2 facilities and practices supplemented by controlled access to the laboratory and use of special laboratory clothing and partial containment equipment (e.g. a biological safety cabinet). Work surfaces are decontaminated after any spill of infectious material or at least once a day. Entering a class 3 area is possible only via a lock system (two self-closing doors) that strictly separates the area from adjoining rooms. Windows must be sealed or constructed in such a way that they cannot be opened. Access is allowed only for authorized and trained persons who have been instructed in the specific risk situation and whose presence is required. All persons should be supervised by competent scientists who are experienced in working with the agents handled in the laboratory. Technical staff need to be accompanied by skilled persons. Laboratory personnel should be immunized against the agents handled or potentially present in the laboratory.
- *Biological safety level 4 (BL-4)* requires even more strict safety practices than BL-3. BL-4 units are usually located in a separate building. Exhaust air must be HEPA filtered. Entrance doors are usually supplied with access control systems and a lock system (three rooms) with a ventilation system that guarantees that the air stream is flowing into the BL-4 area. Laboratories must be separated from common areas in such a way that access is restricted to authorized persons and is impossible for non-authorized persons. A logbook must be used, indicating the date and exact time of entry and exit. The laboratories have to be disinfected before access of other persons (e.g. technical staff) is allowed. Working alone is not allowed. The laboratory worker must be completely protected from aerosolized infectious materials, which is accomplished by working in a class III biological safety cabinet or a full-body, air-supplied positive-pressure personnel suit. All persons have to take a decontaminating shower before leaving the laboratory. A double-door autoclave and a pass-through dunk tank must be available for decontaminating materials passing out of the laboratory.

Housing systems and operational practices

Biosafety criteria for housing vertebrates have been defined in the USA by the Centers for Disease Control (1988) for biosafety levels 2 and 3, and later for all 4 biosafety levels (Centers for Disease Control/National Institute of Health, 1993). Specific regulations for housing infected

animals according to different safety levels also exist in other countries (e.g. for Germany see Gentechnik Sicherheitsverordnung Anhang V). Therefore, only general comments are given here.

Laboratory animal facilities may be organized in different ways. Sometimes, animal facilities are extensions of laboratories and are managed under the responsibility of a research director. Large research institutions, companies or universities often have centralized laboratory animal facilities that are managed by laboratory animal specialists. They are usually separated from laboratories or institutes. Such facilities usually easier fulfil the legal requirements (animal welfare, safety) due to a more proficient management and specialized personnel, and their size. Centralized animal facilities are usually multipurpose with a number of animal species or strains that are used for a variety of different experiments (short or long term) for different scientific disciplines (e.g. toxicology, immunology, biochemistry, pharmacology, teratology, surgery). Several housing systems (conventional units, barrier unit, isolators) or microbiological quality standards (infected, pathogen-free, gnotobiotic) can be found in large facilities (for more details, see pages 130–137). Therefore, strict separation of animals used for different experiments (studies of infectious or non-infectious disease) or purposes (production and breeding, quarantine) is usually self-evident, not only for safety reasons, but also in order to avoid research complications or influences between experiments. Traffic flow in centralized animal facilities is usually reduced to a minimum, thus minimizing the risk of cross-contamination. Such facilities are usually constructed in a way that proper cleaning and personal hygiene is facilitated. Bedding material from animal cages is removed in a way that formation of aerosols is avoided, in order to minimize the risk of allergies and to reduce the risk of airborne transmission of pathogens. Use of solid bottom cages helps to reduce dust formation and is absolutely necessary if experimentally infected animals are housed. The whole facility must be constructed in a way that escape or theft of animals is impossible.

In general, biosafety levels recommended for working with infectious materials *in vitro* and *in vivo* are comparable. Some differences exist, because the activity of the animals themselves can introduce new hazards by producing dust or aerosols, or they may traumatize humans by biting and scratching. Therefore, the Centers for Disease Control/National Institute for Health (CDC/NIH) (1993) established standards for activities involving infected animals, designated 'animal biosafety levels' (ABSL) 1–4. These combinations describe animal facilities and practices applicable to work on animals infected with agents assigned to the corresponding BL1 to BL4.

Housing animals of ABSL-1 is usually no problem if an animal facility as well as operational practices and the quality of animal care meet the standard regulations (CCAC, 1980; Bruhin, 1989; ILAR, 1997). In contrast to experiments with non-infectious materials, additional hygiene procedures should be applied, such as decontamination of work surfaces after any spill of infectious material and decontamination of waste before disposal. Persons who may be at increased risk of acquiring infections

should not be allowed to enter rooms in which infected animals are housed.

Additional practices are necessary for ABSL-2. Careful hand disinfection is necessary after handling live micro-organisms. All infectious waste must be properly disinfected (preferably by autoclaving), and infected animal carcasses should be incinerated. Cages and other contaminated equipment are disinfected before they are cleaned and washed. Whenever possible, infected animals will be housed in isolation to avoid the creation of aerosols. Physical containment devices are not explicitly required by the CDC/NIH (1993) for ABSL-2. Microisolator cages are not recommended because they do not reliably prevent aerosol formation and transmission of micro-organisms. They should only exceptionally be used for housing, and must be placed in ventilated enclosures (e.g. laminar flow cabinets). Therefore, the lowest level of biocontainment should be ventilated cages with negative pressure. In many institutions negative-pressure isolators are considered the only suitable containment devices for housing animals infected with potential human pathogens. Special care is necessary to avoid infections during necropsy of infected animals. Necropsies as well as harvesting tissues or fluids from infected animals should therefore be carried out in safety cabinets.

Like for work with BL3 materials, access to an ABSL-3 facility is highly restricted. Laboratory personnel receive appropriate immunizations (e.g. hepatitis B vaccine). Physical containment devices are necessary for all procedures and manipulations. Animals must be housed in a containment caging system. Individually ventilated cages might be acceptable in specific cases, but negative-pressure isolators or class II biological safety cabinets offer a maximum of safety because supply and removal of infected materials is done in closed containers, thus reliably avoiding a risk of transmission. Very few facilities house ABSL-3 animals. If this is really necessary, much greater safety precautions will be taken than recommended by CDC/NIH (1993) (e.g. one-piece positive-pressure suit ventilated with a life-support system).

ABSL-4 is extremely uncommon and will be avoided whenever possible because transmission of extremely pathogenic organisms to humans is always possible by scratching or biting. Maximum access control and hygiene measures are necessary.

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2 *In Vitro* Analysis

2.1 Isolation and Preparation of Lymphocytes from Infected Animals

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◆◆

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◆◆◆◆◆◆ INTRODUCTION

Lymphocytes are the cells that provide specificity to host defence. Identifying the phenotype and antigen specificity of lymphocytes that have been isolated from animals infected with microbial agents is integral to understanding protective immunity. There are various methods of isolating, purifying and characterizing lymphocytes from the tissues of infected animals. Some of these are elegant and sophisticated procedures that rely on expensive instrumentation (i.e. flow cytometry) to yield highly purified and well-characterized cell populations. These procedures are not the principal subject of this chapter, which will focus largely on simple preparative techniques that can be used by nearly any microbiology laboratory. These techniques will yield populations of lymphocytes suitable for functional assessment *in vitro*, or adoptive transfer to recipient animals *in vivo*. For detailed descriptions of more sophisticated techniques, the reader is referred to specific chapters in this volume dealing with these subjects.

METHODS IN MICROBIOLOGY, VOLUME 25
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◆◆◆◆◆◆ SOURCES OF LYMPHOCYTES

Lymphocytes can be obtained from a variety of tissues of infected animals. The numbers of cells that can be recovered from each site vary depending on the type of tissue, the age and physiological status of the animals, the immune status of the donor, the virulence of the infectious agent, and the time during the infection at which the tissue is sampled. The investigator needs to decide what site is most relevant, convenient and able to yield the required numbers of cells, before initiating a study. For systemic immunization or infection studies, the most common sources of lymphoid cells are the spleen, peritoneal cavity and draining lymph nodes. If the investigator is interested in the mucosal immune response, then relevant sites include the mucosal associated lymphoid tissues (Peyer's patches, etc.), mucosal epithelium and draining lymph nodes.

Thymus

The thymus is relatively large in young animals, and then involutes with age. It is easily removed and disrupted, yielding large numbers of a relatively pure population of T lymphocytes; few B lymphocytes are also present. These T cells are largely immature cells (mostly $CD4^+CD8^+$) that have not yet been primed by exposure to antigen (Ritter and Boyd, 1993).

Spleen

Unlike the thymus, the spleen does not involute as the animal matures. The spleen can be readily disrupted, yielding mixed suspensions of T and B lymphocytes, macrophages, some neutrophils, dendritic cells and stromal cells (fibroblasts). The latter are usually a minority of the cells present. Splenomegaly is common during many types of microbial infection. This increases the numbers of cells that can be isolated, and changes the phenotypes of the cell populations present (Haak-Frendscho and Czuprynski, 1992).

Peritoneal Exudate Cells

The unperturbed peritoneal cavity contains a mixed population of mononuclear cells (both lymphocytes and macrophages). Neutrophils and eosinophils should be present in very low numbers. Mast cells are a minority population (generally 5% or less), but are conspicuous because of their large basophilic granules. After intraperitoneal injection of microbes, microbial products or other sterile irritants, there is a rapid influx of inflammatory cells into the peritoneal cavity. There is temporal sequence to the leukocyte infiltration (Czuprynski *et al.*, 1984). The earliest to arrive are neutrophils, which predominate during the early phase of inflammation (4–12 h). These are later replaced by

eosinophils and mononuclear cells (especially macrophages), although neutrophils continue to be present in significant numbers up to 24 h after injection of irritants such as thioglycollate or proteose peptone. The numbers and types of cells vary depending on the genetic phenotype and immune status of the recipient and the nature of the agent injected. Injection of microbial antigens will elicit little cellular response in a non-immunized animal, whereas it can initiate a significant influx of inflammatory leukocytes in an immunized animal (Czuprynski *et al.*, 1985).

Lymph Node Cells

Lymph node cell suspensions contain B and T lymphocytes, as well as macrophages, dendritic cells and stromal cells. The numbers of lymphocytes recovered from lymph nodes depend on which nodes are collected, and whether there has been recent antigen stimulation. A single mouse lymph node will yield relatively few cells (generally not more than 1×10^6). Thus, recovery of adequate numbers of cells requires careful planning regarding the numbers of animals in each experimental group, and the number of lymph nodes that must be harvested to obtain the numbers of cells required. Lymph nodes contain a tough capsule that must be mechanically disrupted with forceps, or a mesh screen, to release the lymph node cells from the network of stromal cells and extracellular matrix proteins.

Peyer's Patch and Other Mucosal Associated Lymphoid Tissue

If one is interested in investigating the mucosal immune response, it is important to obtain lymphocytes from mucosal-associated lymphoid tissue (MALT) that has been exposed to the microbial agent or its antigens. The specific mucosal site sampled depends on the nature of the infectious agent being studied. Most investigations focus on the gut-associated lymphoid tissue (GALT), since at least some of its lymphoid aggregates (i.e. Peyer's patches, mesenteric lymph nodes) are visible to the naked eye and can be readily removed from the surrounding mucosal tissue (Czuprynski *et al.*, 1996). Similar principles would be followed if working with lymphocytes from other mucosal sites (e.g. respiratory or genitourinary tracts). These large lymphoid aggregates can be excised and mechanically dispersed as described for lymph node cells. In addition to the large lymphoid aggregates in the MALT, there are also intraepithelial lymphocytes (IELs) scattered throughout the mucosa. These can be released simply by incubating the tissue in medium, allowing the IELs to emigrate out of the tissue into the medium (Ishikawa *et al.*, 1993).

The following protocol has been reported for isolation of murine IELs (Ishikawa *et al.*, 1993).

1. Remove the small intestine and flush out the lumen contents. Invert the intestine with a piece of polyethylene tubing, and then cut it into three or four segments. Transfer up to 10 segments to a plastic box containing 250 ml RPMI-1640 tissue culture medium with 2% fetal bovine serum (FBS), 25 mM *N*-[2-hydroxyethyl]piperazine-*N*-[2-ethanesulphonic acid] (HEPES) and penicillin streptomycin (100 units ml^{-1} and 100 μg ml^{-1}, respectively).
2. Place the box on an orbital shaker (150 rpm) in a 37°C incubator for 45 min.
3. Remove the non-adherent cell suspension and pass through a glass-wool column to remove debris and adherent cells (i.e. macrophages and stromal cells).
4. Add the non-adherent cells to a discontinuous Percoll gradient (44% and 70%) and centrifuge for 30 min at 400*g*. The IELs are removed from the gradient interface and washed twice in RPIM-1640 with 2% FBS.

◆◆◆◆◆◆ ISOLATION OF LYMPHOCYTES FROM TISSUES

One can use both mechanical and enzymatic techniques to disrupt and release lymphocytes from normal tissue architecture. In most instances, the lymphocytes remain non-adherent and can be easily removed from the larger and more dense parenchymal and stromal cells. The lymphocyte-enriched cell suspensions then can be purified further to obtain the cells needed. Specific procedures that can be used to release tissue lymphocytes are described below.

Mechanical Disruption

Tissue can be mechanically disrupted using several methods. The simplest is to cut the tissue into conveniently sized fragments, and then push these through a sterile nylon strainer using sterile forceps or the shaft of a plastic syringe. Some investigators prefer to tease the tissue apart using sterile forceps and scissors. With either procedure, the concept is the same: break open the tissue capsule, disrupt the stromal architecture and release the lymphocytes.

In the past, investigators frequently relied on the use of narrow mesh stainless steel screens for tissue disruption. These are effective, but present challenges regarding cleaning and sterilization. It is more convenient to use the disposable sterile nylon mesh screens that can be purchased from commercial suppliers (Falcon No. 2340, Becton Dickinson, Bedford, MA, USA). The lymphoid tissue (e.g. spleen or lymph node) is rubbed across and pushed through the screen with a syringe plunger into a small plastic

dish that contains tissue culture medium or balanced salts solution. The cell suspensions are then washed several times to remove debris and used as an unseparated cell suspension, or subjected to further purification as needed.

◆◆◆◆◆◆ CELL SEPARATION

Use of Density Gradients

The use of density gradients was initially described by Boyum (1968). Employing a mixture of Ficoll (Sigma No. 8016, St Louis, MO, USA) and sodium diatrizoate (Hypaque, a radiocontrast medium, Sigma No. S-4506), one can obtain gradients of specified density and osmolarity suited to the separation of cell types based on buoyant density. The specific density of the gradient that is needed for successful cell separation depends on the species and cell type of interest. For example, human peripheral blood cells can be purified from diluted whole blood (using phosphate buffered saline (PBS) as diluent, generally at 2 or 3 parts per volume of blood) using Ficoll-Hypaque of 1.077 density (290 mM osmolarity, Sigma No. 1077-1). This can also be used for mouse peritoneal or spleen cells, although some find that a 1.083 (320 mM osmolarity) density Ficoll-Hypaque ('Lympholyte-M', No. ACL-5030, Accurate Chemical, Westbury, NY, USA) is better suited for this purpose. Determining the optimum density for a particular cell from a given species is an empirical process. Starting with a 1.081 density medium, and centrifuging at 400*g* for 30 min at room temperature is a good starting point. By altering the density, time and centrifugal force, a satisfactory separation can be achieved for many cell types.

The following procedure can be used to isolate murine lymphoid cells.

1. Suspend the cells in calcium and magnesium-free PBS or HBSS, with 10 mM ethylenediaminetetraacetic acid (EDTA) added to prevent cell clumping or fibrin deposition. The cells should preferably be placed in a round-bottomed rather than a conical disposable plastic polystyrene tube.
2. Using a long Pasteur pipette, or sterile needle with a syringe, underlay the diluted cell suspension with 0.5 ml Ficoll-Hypaque per millilitre of cell suspension. This should be added slowly, to avoid mixing.
3. The tubes should be centrifuged at 400*g* for 30 min at 22°C. The *G* force needed will vary depending on the density of the Ficoll-Hypaque, and the type of cells being isolated. One should consult a nomograph for your centrifuge to determine what speed is needed to obtain the desired *G* force. It is important that the centrifugation be done at 22–25°C, as the density of the gradient changes at refrigeration temperature (4–10°C).

4. The mononuclear cells (lymphocytes and mononuclear phagocytes) will accumulate as a hazy white band at the gradient interface, from which they can be readily aspirated.
5. Granulocytes (principally neutrophils) can be recovered from the cell pellet. The pellet can also contain erythrocytes, mast cells, eosinophils, immature leukocytes and, possibly, stromal cells.
6. Contaminating red cells can be lysed with dilute ammonium chloride (8.29 g NH_4Cl, 0.37 g Na_2 EDTA, and 1 g $KHCO_3$ in 1 l distilled H_2O, pH 7.3). In some instances (i.e. bovine blood), red cells can be lysed by a brief hypotonic shock using dilute phosphate buffer (37 mM phosphate, pH 7.20, no sodium chloride) for 45 s, before restoring isotonic conditions (by adding 0.1 volume 8.5% NaCl buffered with 37 mM phosphate, pH 7.20).
7. Both the mononuclear and granulocyte populations should be washed two or three times in Ca^{2+} and Mg^{2+} containing Hank's balanced salts solution (HBSS), or tissue culture medium, to remove the contaminating Ficoll-Hypaque before the cells are used. Failure to do so could result in loss of viability or functional activity, as the Ficoll solution is somewhat toxic to cells.
8. Contamination with bacterial endotoxin can occur (Haslett *et al.*, 1985), which is a concern in many cell culture systems. Each investigator should check their stocks of reagents, and the distilled water used to prepare them, with the *Limulus* assay (Biowhitakker, Walkersville, MD, USA) to estimate levels of endotoxin contamination.

An alternative density gradient procedure involves the use of Percoll, a polyvinylpyrrolidone coated colloidal silica in water. Percoll can be used to produce discontinuous or continuous density gradients, which can be used in a manner similar to Ficoll-Hypaque, to isolate various types of leukocytes (Harbeck *et al.*, 1982).

Use of Adherence to Remove Mononuclear Phagocytes

The tendency of mononuclear phagocytes to adhere to glass or tissue culture plastic, can be used to enrich for, or remove, macrophages from a mononuclear cell suspension. Mononuclear phagocytes obtained from different tissue sites are all adherent, but can differ in how strongly they adhere. In general, cells activated at sites of inflammation *in vivo* are more adherent than resident cells. The purity of the lymphocyte populations obtained after adherence can vary, depending on the type of tissue culture grade plastic used, and the presence or absence of additional coating (e.g. serum proteins, poly(L-lysine)) on the surface. However, commonly avail-

able tissue culture plates have all been designed to promote adherence, so that the differences among manufacturers should not pose significant problems in many applications. Adherence can be performed with or without the addition of serum or other proteins (e.g. bovine serum albumin (BSA)). In general, mononuclear phagocyte adherence is higher in the absence of serum, but non-specific adherence of lymphocytes is also greater (Musson and Henson, 1979).

A general outline for depletion of mononuclear phagocytes, to yield lymphocyte-enriched cell populations is described below (Czuprynski *et*

1. The mononuclear cells are plated onto tissue culture flasks at a density not exceeding 2×10^7 cells per 25-cm^2 flask, or 2×10^6 to 4×10^6 cells ml^{-1} (or per well), in a 24-well tissue culture cluster plate.
2. The cells are incubated at the appropriate temperature (usually 37–39°C) with 5% CO_2 for 1–2 h.
3. To recover a mononuclear-phagocyte-depleted population of cells (i.e. largely lymphocytes), the non-adherent cells are gently removed by carefully decanting them into a new tissue culture flask. A second round of adherence can be performed, if needed, to try to remove additional mononuclear phagocytes.
4. The non-adherent cells are then centrifuged, counted with a haemocytometer and checked for viability (see later section on viability and counting cells).
5. To confirm depletion of mononuclear phagocytes, a cytocentrifuge smear is prepared on a clean glass slide and a differential stain performed (Wright–Giemsa, or Diff-Quik). The esterase stain is a more specific staining technique, mononuclear phagocytes will usually stain darkly, whereas lymphocytes will be negative or exhibit a discrete spot of staining (Koski *et al.*, 1980).
6. Mononuclear-phagocyte depletion can be verified by staining with a fluorochrome-conjugated antibody directed against a monocyte/macrophage-specific cell surface antigen (i.e. F4380 in the mouse) (Springer, 1981), followed by flow cytometry or fluorescent microscopy. Assessing ingestion of latex beads, or opsonized yeast, is an additional functional assay, that can be performed to identify phagocytes.
7. To recover a mononuclear-phagocyte-enriched population, the adherent cells can be removed physically with a plastic cell scraper after two or three *gentle* washes of the adherent cells with 10–15 ml warm medium.
8. Alternatively, the adherent cells can be removed from the flask surface by incubation for 5–10 min at 37°C with 0.1% trypsin in Ca^{2+} and Mg^{+2} free PBS or HBSS with 10 mM EDTA. Because trypsin treatment also removes proteins from the cell surface, caution must be used when staining for surface antigens or performing functional assays.

al., 1983). To perform adherence, mononuclear cells are washed two or three times in HBSS or tissue culture medium.

Differential staining (Wright–Giemsa or Diff-Quik) and estimation of viability (i.e. Trypan Blue exclusion) should always be performed. If there is a significant percentage of non-viable cells, these can be removed by pelleting the cell suspension through a Ficoll gradient, as described earlier. The viable mononuclear cells will remain at the gradient interface, whereas the dead cells will pellet at the bottom of the gradient.

Use of Nylon Wool to Enrich for T Lymphocytes

Nylon wool has long been used as a rapid preparative method to enrich T cells from complex mixtures of cells such as bone marrow, peripheral blood and spleen (Julius *et al.*, 1973). The technique takes advantage of the

1. The nylon wool (Polysciences, Warrington, PA, USA) is pretreated, to remove toxic impurities, by boiling for 1 h in distilled water which has been made 0.2 N in HCl. The wool is then rinsed extensively with tissue culture grade (18 MΩ) water by boiling for 20 min, in three successive changes of water.
2. The nylon wool is dried, packed loosely (to the 8-ml mark) in 10-ml polypropylene syringes, wrapped and autoclaved. A nylon-wool column so prepared is sufficient to allow T-cell enrichment from a starting cell suspension of no more than 5×10^7 cells. The nylon wool can be removed from the column and reused by following the cleaning procedure outlined above.
3. To enrich for T lymphocytes, resuspend up to 5×10^7 mononuclear cells in 1.0 ml HBSS with 5% fetal bovine serum (HBSS-FBS). The columns should be washed with at least 35 ml of warm medium before the cells are added in a volume of 1–2 ml. The cells are washed into the column with an additional 2 ml of warm medium, and the column incubated in a 37°C incubator for 45 min.
4. The T-lymphocyte-enriched cell suspension is eluted by slowly washing the column with 20 ml of warm HBSS-FBS. The eluted cells are then centrifuged and resuspended at an appropriate concentration in the medium of choice (usually RPMI-1640 or Dulbecco's modified Eagle medium (DMEM).
5. The efficacy of macrophage depletion should be verified by microscopic examination of cytocentrifuge prepared smears that are differentially stained with Wright–Giemsa or Diff-Quik. Monocyte contamination can be determined by esterase staining, and T-cell purity by fluorescent microscopy or flow cytometry, using FITC-Thy1 monoclonal antibody (mAb).
6. Usually one can expect to obtain approximately 60–80% T cells (Julius *et al.*, 1973; Czuprynski *et al.*, 1985). Multiple rounds of nylon wool passage can improve this somewhat.

relative 'stickiness' of mononuclear phagocytes, and B cells, which tend to adhere when passed slowly through a column (usually a 10-ml syringe) of loosely packed nylon wool.

Use of Complement-mediated Lysis to Remove Cell Populations

Complement-mediated lysis provides a powerful and convenient method for eliminating specific cell populations (Hathcock, 1991). The chief limitation is that you must use an antibody, specific for the surface marker of interest, of an isotype that can fix complement. Lymphocytes are suspended in RPMI (or HBSS) containing 5% FBS, at a cell concentration ranging from 1×10^7 to 1×10^8 cells ml^{-1}. The total volume should be kept small in order to conserve the amount of antibody used. The antibody used can be a polyclonal or monoclonal, and either purified or unpurified (ascites, antiserum or culture supernatant). Appropriate specificity controls (an antibody of the same isotype that does not bind to the cell type of interest), must be included in all cases. If the antibody concentration, and complement-fixing activity are not known, these should be titrated beforehand, to determine the minimum amount needed for effective lysis.

If the antibody for the marker of interest does not fix complement, then a complement-fixing second antibody, which specifically recognizes the heavy or light chains of the first antibody, can be used. Concern about complement-fixing ability is less of an issue when using polyclonal antibodies, since immunized rabbits or goats will generally produce some antibodies that bind complement.

The complement source is important. Mouse serum is generally low in complement lytic activity (Ooi and Colten, 1979), and hence a homologous system cannot be used. Baby rabbit serum (Low-Tox M, Accurate Chemical, Westbury, NY, USA) is an excellent source of high-titre complement, which generally works well with mouse and rabbit antibodies. Guinea-pig complement also usually works well. It may be necessary to absorb the complement source with agarose (see below) to remove non-specific toxicity before it is added to the antibody-coated cell suspension (Cohen and Schlesinger, 1970). Appropriate controls include use of an irrelevant antibody of the same isotype, plus the complement source, to demonstrate specificity of cell lysis.

To absorb rabbit or guinea-pig serum for use in complement depletion, use the following procedure.

1. Thaw the serum on ice.
2. Add 1 g molecular-biology grade agarose per 10 ml serum.
3. Incubate on ice, with occasional mixing, for 15 min.
4. Centrifuge at 400*g* and carefully remove the supernatant. Store the absorbed serum in aliquots at –70°C. To prevent loss of lytic activity, avoid freeze–thawing, and keep on ice until used in an experiment.

The following protocol could be used to perform complement-mediated depletion of a T-cell subset:

1. Suspend mononuclear cells in RPMI containing 5% FBS at a cell concentration of 5×10^6 to 50×10^6 ml^{-1}.
2. Include a control antibody tube (same isotype that does not bind the cells of interest), and a complement-only control tube. Add the primary antibody at 1-2 μg per 10^6 cells (it is important to use a concentrated preparation of antibody to avoid excessive dilution of the cells).
3. Incubate on ice for 45 min, wash twice with media, and resuspend the cells in the original volume of medium.
4. If the primary antibody does not fix complement, add 1–2 μg of the second antibody (which recognizes the light or heavy chains of the first antibody), and incubate 30 min on ice.
5. Wash the cells twice with ice-cold RPMI-FBS, and resuspend in the original volume of the same.
6. Add an equal volume of the complement source (diluted to an appropriate concentration in medium) and incubate in a 37°C water bath for 30–45 min.
7. Wash the cell suspension twice with medium, and verify depletion by staining with FITC-labelled antibody for the marker of interest, followed by flow cytometry or fluorescent microscopy.

If cell depletion is not satisfactory, a second round of complement-mediated lysis can be performed. If the cells exhibit clumping, strain through a 40-μm nylon screen, or gently pipette up and down through a 5-ml pipette, to disperse the cell clumps. This is particularly important if the cells are to be transferred to recipient animals, as intravenous injection of cell clumps can cause thrombosis and death.

Use of Magnetic Beads

The use of magnetic beads coupled to specific antibodies is a rapid and powerful technique to obtain highly purified cell subsets. The technique requires mAbs against cell surface markers of interest, magnetic-bead-coupled second antibodies and a high-flux-field magnet (Monk *et al.*, 1990). The latter can be in the form of either a magnetic plate that is placed underneath a flask or tissue culture plate (Becton-Dickinson, Bedford, MA, USA), or a magnetic column through which the cells pass (Miltenyi Biotec, Auburn, CA, USA).

For a detailed description of these procedures, the reader is referred to the appropriate chapter (1 in Section I). We have used the magnetic plate as a bulk method for selecting or depleting cell types from a mixed cell suspension. In brief, the cells are suspended in RPMI-FBS, or other appropriate medium, incubated with the mAb of interest (1–3 μg per 10^6 cells) in a small volume (0.2 ml) on ice for 45 min. The cells are washed twice with cold medium, and resuspended in the original volume of fresh medium. An

appropriate magnetic-bead-conjugated second antibody is then added, and the cells incubated for 45 min on ice (to prevent capping). The resulting cell suspension is then placed in a small tissue culture flask, which is placed on a magnetic plate (Collaborative Research, Bedford, MA, USA) and incubated at room temperature for 15 min. Those cells expressing the marker of interest will quickly settle to the bottom of the flask, where they will be held by the magnetic force exerted on the cell-associated beads. The negative (non-adherent) cells are decanted to a sterile tube or flask, washed and then resuspended in medium before being tested for viability and depletion of the subset of interest. Although recovery of the positively selected population can be done by removing the plate from the magnet, the magnetic beads must drop off before the cells can be used in a functional assay. In our experience the efficacy of the method depends on the surface marker and mAb being used. Each investigator should verify their own experimental system.

Alternatively, antibody-labelled cells can be passed through a cell-permeable matrix in the presence of a high-flux magnet (Miltenyi Biotec, No. 42201, Auburn, CA, USA) (Monk *et al.*, 1990). This method can yield a high purity negatively-selected population; recovery of the positive cell population is also possible. The method is well suited to the purification of large numbers of cells, since several columns can be employed simultaneously, and sequential passages through the column (using different mAbs) can be performed in a few hours. Incubating the cells for 16–24 h *in vitro*, facilitates shedding of the antibody-counted beads that are used in the separation process.

Panning

Petri dishes or plastic flasks coated with an antibody against a particular cell surface marker can be used to immobilize that cell type on the plastic surface ('panning') (Wysocki and Sato, 1978). The technique works best for negative selection, but can be used for positive selection as well.

1. Use bacteriologic plastic Petri dishes to reduce non-specific adherence of cells. Coat the dishes with 4–5 ml of a solution (150 mM NaCl with 50 mM Tris buffer, pH 9.5) containing antibody (10 μg ml^{-1}) specific for the surface marker of interest, and incubate overnight at 4°C.
2. Remove the fluid and wash the dish four times with PBS/1% FBS. Block non-specific binding sites by incubating the dish with PBS/1% FBS for 30 min at room temperature.
3. Wash the plate four times with HBSS/1% FBS. Add 20×10^6 to 200×10^6 cells per dish (depending on cell type) in 5 ml cold HBSS/1% FBS.
4. Incubate the dish at 4°C for 1 h. Gently aspirate the medium and remove the unattached cells. Wash twice with 3–5 ml cold HBSS/1% FBS.
5. Centrifuge the cells at 4°C. Wash twice with the medium that will be used in subsequent experiments.

Use of Flow Cytometry

Flow cytometric cell sorting can yield very high purity cell preparations. For a detailed description of this method the reader is referred to the book by Shapiro (1988) and to chapter 1 in Section I. The method involves labelling the subset of interest with a fluorochrome-labelled primary or secondary antibody, and then sorting the cells after gating for cell density and fluorescence. Cells (5×10^6 to 50×10^6) are incubated with 1–3 µg antibody per 10^6 cells in a small volume (0.1–0.2 ml), at 4°C to prevent capping. Sorting of two or more cell populations can be achieved using multicolour staining with different antibodies, each of which recognizes a distinct surface marker and is conjugated with a different coloured fluorescent probe. The drawbacks of cell sorting include the time needed for sorting, the difficulty of maintaining sterile cell suspensions and the limitations on the numbers of cells that can be sorted. For these reasons, the less sophisticated preparative methods already described (columns, adherence, lysis, etc.) are often used, at least initially, when large numbers of cells are required.

◆◆◆◆◆◆ QUALITY CONTROL

Once a cell suspension has been obtained it is important that it is assessed for the purity of the cell populations present and the viability of those cells. There are various ways this can be done. We briefly discuss some of the simpler and more rapid methods.

Viability

Trypan blue

Probably the most frequently used rapid method for assessing cell viability is the exclusion of Trypan Blue dye. Viable cells actively transport Trypan Blue out of the cell and remain refractile and colourless. In contrast, cells that are dead or have a damaged cell membrane cannot eliminate Trypan Blue and will appear pale to dark blue (Caron-Leslie *et al.*, 1994). Although rapid, easy and inexpensive, the method suffers from several limitations. First among these is the question of whether blue cells are truly dead or merely exhibit membrane damage that might be reversible. Secondly, if cells have been killed and completely lysed, then enumerating only the percentage of cells that take up Trypan Blue may grossly underestimate cell death, unless one incorporates a total cell count. A protocol for performing Trypan Blue exclusion is given below.

1. Make up 0.4% Trypan Blue in isotonic saline.
2. Add one drop per 0.2 ml of cell suspension.
3. Load into a haemocytometer and count at least 100 cells at 100× magnification. Score for both blue (dead) cells, and the total number of cells.
4. Calculate the percentage of dead cells, and the total number of cells present.

Propidium iodide staining

Propidium iodide is also excluded from viable cells. Dead cells become permeable to the dye, which then intercalates with their DNA. The resulting staining can be detected by flow cytometry or fluorescence microscopy (Shapiro, 1988). Using proper gating for the cell population of choice, this can provide a useful estimate of the proportion of dead cells in a cell suspension.

Vital dye staining

Some dyes (e.g. Neutral Red) are only taken up by viable cells. These dyes can be used to stain the cells, and an estimate of the viability of the cell population (monolayer or cell suspension) can be made by using the relative light absorbance (Kaufmann *et al.*, 1987). Inclusion of appropriate controls for background lysis, and maximum cell lysis, allows generation of standard curves from which one can extrapolate the number of viable cells.

Reduction of tetrazolium salts

Viable cells will take up various tetrazolium salts: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2,5-bis[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT). These compounds are reduced to a formazan compound in the mitochondria of intact cells. This formazan product can be readily detected with a spectrophotometer, thus providing a means of measuring both cell viability and metabolic activity (Green *et al.*, 1984; Scudiero *et al.*, 1988). For cells that proliferate (lymphocyte blast transformation), MTT or XTT provide an attractive alternative to the use of radioisotopes such as [^{3}H]thymidine. For cells that are not proliferating, the absence of signal (i.e. decreased dye reduction) is an indication of cell death, or at least physiological inactivity. Inclusion of proper controls for background and maximal cell lysis, allows estimation of the percentage of viable cells.

1. Add cells (2×10^6 ml^{-1}) to wells of a 96-well microtitre tissue culture plate.
2. Prepare XTT (1 mg ml^{-1}) in warm medium without sera, and phenazine methosulphate (PMS) at 5 mM (1.53 mg ml^{-1}) in PBS.
3. Add PMS solution (1 : 20 by volume) to the XTT solution to make 0.25 mM PMS-XTT (both from Sigma, St Louis, MO, USA) solution.
4. Remove the medium from the cells and replace with the 0.25 mM PMS-XTT solution. Incubate for 4 h at 37°C. It might be necessary to incubate resting cells, that are metabolically less active for a longer period.
5. Mix the medium, and read the absorbance at 450 nm using a micro enzyme linked immunosorbent assay (micro-ELISA) plate reader (e.g. Dynatech Model 600).

Differential Staining

The types of cell present in a cell suspension can be estimated by staining the cells with one of several commercial stains. If the cells are adherent to a glass or plastic surface (i.e. coverslip), as for macrophages, this is a simple matter. If the cells are in suspension or non-adherent (as for lymphocytes), then it is first necessary to adhere the cells mechanically to a clean glass slide before they are stained. To do this, one adds a small volume (usually 0.1 ml) of a cell suspension at an appropriate density (usually 0.5×10^6 to 2×10^6 ml^{-1}) to a chamber in a cytocentrifuge (Shandon-Lipshaw, Sewickley, PA, USA). The centrifugal force exerted during operation forces the cells onto the surface of the slide, where they form a circular ring that is readily visible. The slide is then air-dried, fixed with methanol (by air drying or with ethanol) and stained with Wright–Giemsa or Diff-Quik stains. With a little training, these cells can then be examined microscopically at 400× magnification and scored for cell type (e.g. lymphocyte, neutrophil, macrophage). When so doing, at least 200 cells on each slide should be scored, and the results expressed as the percentage of each cell type in the total cell population. The absolute cell number should also be determined using a haemocytometer or Coulter counter and the results expressed as the absolute number of each cell type.

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2.2 Establishment of Murine T-cell Lines and Clones, Hybridomas and Transfectomas

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INTRODUCTION

Most experimental systems benefit from simplicity. This certainly applies to studies of the immune responses where complex cellular interactions often make it extremely difficult to understand and appreciate the mechanisms underlying a particular effect. Early on, the need for simplified experimental systems has led to the cloning of targets of the immune response which, as transformed tumour cells, were more amenable to tissue culture. However, the need for simplicity is even more apparent in analyses of the responding cells of the immune system. The fact that both B and T cells express clonally distributed antigen receptors, and therefore carry clonal specificities, necessitates clonal analyses in studies on their responses. This first led to the development of cloning systems for B cells and their antibodies (Köhler and Milstein, 1975a,b; Shulman *et al.*, 1978; Kearney *et al.*, 1979). Similar approaches for T cells were delayed, mostly because our understanding of T cells has developed more slowly (Morgan *et al.*, 1976; Goldsby *et al.*, 1977; Kappler *et al.*, 1981; White *et al.*, 1989).

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Cloning of T cells may have been even more crucial in attempts to understand their functions. Because T cells express their antigen receptors on their cell surface instead of secreting them, studies of T-cell specificity more often depend on the isolation of the reactive cells themselves.

Cloning does not only represent an approach to studying individual T cells, however. Collections of clones, mostly in the form of hybridomas, have been used as representatives of mixed cell populations, for T-cell receptor (TCR) repertoire studies and for following the development of an immune response. Furthermore, cloned T cells readily permit one to establish correlations between separately encoded properties, such as cytokine secretion and the expression of cell surface markers, or the pairing of chains in heterodimeric TCRs.

However, approaches to cellular analysis by cloning also suffer from inherent problems. Perhaps the most troublesome is the lack of control over selective forces *in vitro* which affect the population of clones that survive. Secondly, cloned cells, and especially hybridomas, are often unstable (Johnson *et al.*, 1982). Instability combined with non-physiological selection pressures can lead to misrepresentation because of changes in specificity and loss, or occasionally even gain of cellular functions. Thirdly, cloning has not been universally successful. Some types of T cells have defied all attempts at cloning, without clear evidence as to how they are different.

Methods of cloning T cells have become rather sophisticated. However, recently developed techniques that provide access to individual cells in different ways have gained in relative importance. Cell sorting has become increasingly efficient, often obviating the need for cell cloning. TCR transgenic mice permit studies on normal cells with uniform specificities *in vivo*, without the vagueries of *in vitro* systems. (It should be noted, however, that the generation of most TCR transgenic mice is preceded by a cloning experiment to identify and make available cells of desired specificities.) Cellular clones are no longer needed to provide large quantities of cytokines or cell surface molecules, because of the development of molecular cloning systems that permit not only gene expression but also the production of large quantities of proteins with normal or nearly normal post-translational modifications.

◆◆◆◆◆ T-CELL LINES AND CLONES

Long term *in vitro* culture of normal lymphocytes became a possibility with the improvement of tissue culture conditions in the 1970s. Particularly important was the discovery that supernatants from stimulated short-term lymphocyte cultures contain growth factors for T cells (Morgan *et al.*, 1976). The first well-defined T cell growth factor (TCGF) is now known as interleukin-2 (IL-2) (Aarden *et al.*, 1979). Further factors directly or indirectly influencing the growth of T cells *in vitro* are IL-1, IL-4, IL-7, IL-15, γ-interferon (IFN-γ), and various others (Coligan *et al.*, 1993; Okazaki *et al.*, 1989; Nishimura *et al.*, 1996).

Most commonly, instead of purified growth factors, mixtures of growth factors produced in other cell cultures are used to support long-term growth of T cells. Such conditioned media may be derived from mitogen- or alloantigen-stimulated cultures or from certain tumour cell cultures. Different cell cultures produce different mixtures of growth factors, and consequently may support preferential growth of different T-cell subsets. For example, secondary mixed lymphocyte cultures with mouse splenocytes abundantly produce IL-4 and IFN-γ but little IL-2, whereas concanavalin A stimulated spleen cells preferentially produce IL-2 (Gajewski *et al.*, 1989a,b).

Although long-term lines and clones of T cells were derived originally by culture in the presence of growth factors without antigen stimulation (Haas *et al.*, 1985), this approach is now used only if the antigen is not known, as is the case, for example, with most γδ T cells (Tsuji *et al.*, 1996). T-cell culture in the presence of antigen and antigen-presenting cells (APCs), together with or alternating with growth factor stimulation, has proved far more reliable in generating long-term lines and clones with relatively normal features (Fathman and Hengartner, 1978; Glasebrook and Fitch, 1980; Ziegler and Unanue, 1981; Chestnut *et al.*, 1982; Johnson *et al.*, 1982; Kappler *et al.*, 1982). Requirements are different for growing alloreactive and conventional antigen reactive αβ T cells and for cytotoxic T lymphocytes (CTLs) and T-helper 1 and 2 cells (Th1, Th2) (see below). However, in all cases, antigen stimulation is required for long-term growth. Requirements for the culture of the far slower growing γδ T cells are not yet well established.

The description given below of culture conditions for the development and cloning of long-term αβ T-cell lines closely follows the more detailed protocols reported by Fitch and Gajewski (Coligan *et al.*, 1993). With minor modifications, these methods seem applicable to the cloning of murine αβ T cells in general. By comparison, the culture conditions given for γδ T-cell clones are less well established and may not be generally applicable (Tigelaar *et al.*, 1990; Tsuji *et al.*, 1994).

Alloreactive Th and CTL Clones

To obtain the highest frequencies of responding T cells, freshly isolated cells are first stimulated in primary allogeneic mixed lymphocyte bulk cultures (MLCs), and then cloned by limiting dilution.

1. Prime alloreactive T cells *in vitro* (e.g. mix 2.5×10^7 responding mouse spleen cells with an equal number of irradiated (2000 rad) stimulating spleen cells in 20 ml supplemented DMEM-5 medium; culture in an upright 50 ml plastic culture flask for 10–14 days at 37°C, 5% CO_2).
2. Prepare secondary MLC by mixing 6×10^6 washed cells from the primary culture with 2.5×10^7 irradiated splenic stimulator cells; incubate for 36–48 h under the same conditions as for the primary culture.

3. Prepare cloning by plating 10^6 allogeneic spleen cells (2000–3000 rad) in 0.1 ml supplemented DMEM-20 per well of 96-well flat-bottomed microtitre plates.
4. Suspend T cells derived from the secondary MLC in conditioned medium (see Notes below) at 1–10 cells ml^{-1}. Add 50 µl per well of this cell suspension to the prepared cloning plates. Prepare several plates with varying numbers of responding cells to account for possible differences in plating efficiencies. Incubate cloning plates for 4 days under the same conditions as for the primary MLC.
5. Add 50 µl conditioned medium and 50 µl supplemented DMEM-20 to each well. Culture for 7–10 days until clusters of cells become evident.
6. Screen for cytolytic activity or for proliferation.
7. To maintain the desired clones, transfer up to 10^5 cells in 100 µl fresh DMEM-20 from the original microwell to a macrowell in a 24-well microtitre plate containing 6×10^6 irradiated allogeneic spleen cells in 0.9 ml supplemented DMEM-20. Add 0.5 ml conditioned medium, to reach a final volume of 1.5 ml. Passage cells at weekly intervals.

Notes: For the cloning of alloreactive αβ T cells, conditioned medium is best derived from allogeneic primary MLCs (as described above). The supernatant from the C57BL/6 anti-DBA/2 response is particularly rich in the required co-factors. The heat-inactivated culture supernatant is used at a concentration of 20% (v/v) for cloning. Using responder cells from TCR-β "knockout" mice, the same conditions may be applicable for the generation of alloreactive γδ T cell clones (A. Mukasa, unpublished).

Th Clones Reactive with Soluble Protein Antigens

The isolation of such clones requires prior sensitization *in vivo*. Typically, mice are immunized with soluble protein antigens in complete Freund's adjuvant. *In vitro*, syngeneic spleen cells are used as APCs. The following protocols briefly describe the generation of Th1 and Th2 clones reactive with chicken ovalbumin, as described in more detail by Fitch and Gajewsky (Coligan *et al.*, 1993).

1. Immunize mice by subcutaneous injection of antigen (1:1 emulsion of antigen dissolved in Dulbecco's PBS and Freund's complete adjuvant).
2. After 1 week, remove the draining lymph nodes and prepare a single-cell suspension in Hank's balanced salt solution (HBSS) or Dulbecco's modified Eagle's medium (DMEM).
3. Culture 2×10^6 lymph node cells for 6–8 days (37°C, 5% CO_2) in the presence of 6×10^6 irradiated syngeneic spleen cells plus antigen in 1.5 ml supplemented DMEM-5 medium (24-well microtitre plate). For stimulation *in vitro*, protein antigens are typically used at concentrations of 50–400 µg ml^{-1}.

Th1 clones

4a. Prepare 96-well flat-bottomed microtitre plates by adding (per well): 50 µl irradiated syngeneic spleen cells (10^6 per well) in supplemented DMEM-5; 50 µl antigen (0.2–1.6 mg ml^{-1}) in the same medium; 25 µl human rIL-2 (80 U ml^{-1}) and 25 µl mouse rIFN-γ (4000 U ml^{-1}).

5a. Suspend T cells to be cloned at approximately 2000 cells ml^{-1} in supplemented DMEM-5; add 50 µl to each prepared well from step (4a). Incubate for 1 week at 37°C, 5% CO_2. (Because of variable plating efficiencies, T cells should be titred so that plates with 100, 30, 10 and 3 cells per well are generated.)

6a. To each well, add 25 µl human rIL-2 (80 U ml^{-1}) and 25 µl mouse rIFN-γ (4000 U ml^{-1}). Incubate for one additional week or until bottoms of positive wells are almost covered with cells. Wells that have a single cluster of growing cells should be selected for expansion.

Th2 clones

4b. Prepare 96-well flat-bottomed microtitre plates by adding (per well): 50 µl irradiated syngeneic spleen cells (10^6 per well) in supplemented DMEM-5; 50 µl antigen (0.2–1.6 mg ml^{-1}) in the same medium; 25 µl human rIL-2 (40 U ml^{-1}) or 50 µl ConA-sup (40%). Note that some Th2 cells require IL-1 for their growth.

5b. Suspend T cells to be cloned at approximately 2000 cells ml^{-1} in supplemented DMEM-5; add 50 µl to each prepared well from step (4b). Incubate for 1 week at 37°C, 5% CO_2. (Because of variable plating efficiencies, T cells should be titred so that plates with 100, 30, 10 and 3 cells per well are generated.)

6b. To each well, add 50 µl human rIL-2 (40 U ml^{-1}) or 50 ml ConA-sup (10% final). Incubate for one additional week or until the bottoms of the positive wells are almost covered with cells. Wells that have a single cluster of growing cells should be selected for expansion.

Maintenance of the Th Clones

7. Add to each well of a 24-well microtitre plate (final volume 1.5 ml): 5×10^4 to 2×10^5 cloned cells in 100 µl supplemented DMEM-5; 6×10^6 irradiated syngeneic spleen cells in 0.9 ml supplemented DMEM-5; 50–400 µg ml^{-1} antigen; and, for Th1 clones, human rIL-2 (25 U ml^{-1}) and mouse rIFN-γ (250 U ml^{-1}), and for Th2 clones, human rIL-2 (25 U ml^{-1}). (For long-term cultures, use human rIL-2 at a final concentration of 10 U ml^{-1}.)
8. Culture cells at 37°C, 5% CO_2, in a humidified incubator. Passage every 7–10 days.

(*Note*: There are many variations to this basic protocol. For more detail, consult Coligan *et al.* (1993).)

Protective αβ T-cell clone from *Plasmodium berghei* sporozoite-immunized mouse

Balb/c mice were immunized intravenously with *Plasmodium berghei* sporozoites (Tsuji *et al.*, 1990). Responder cells from immunized mice were prepared from NH_4Cl treated splenocytes, by removing B cells on anti-mouse immunoglobulin (Ig) coated Petri dishes. Enriched T cells were adjusted to 3×10^6 cells ml^{-1} of culture medium, and plated at 1ml per well of a 24-well tissue culture plate. To generate APCs, NH_4Cl treated splenocytes of naive Balb/c mice were adjusted to 1×10^7, pulsed with antigen (1 h, 37°C), washed, irradiated (3300 rad) and plated at 5×10^6 cells per well.

Responder and stimulator cells were incubated for 5–6 days in RPMI 1640 culture medium with the usual additives plus 0.5% normal mouse serum, but without fetal calf serum (FCS). Thereafter, antigen-stimulated T cells were purified with lympholyte M, resuspended at 3×10^6 per well (2 ml culture volume), and incubated for 2–3 days in RPMI 1640 culture medium supplemented with 10% FCS, prior to re-stimulation with antigen-pulsed APCs.

From such bulk cultures, a $CD4^+$ cytolytic T-cell clone (A1.6) was derived by limiting dilution. This clone produces IL-2 and IFN-γ *in vitro*, and recognizes a plasmodial antigen in the context of the class II $I\text{-}E^d$ molecule. Passive transfer of this clone into naive mice resulted in a high degree of protection against sporozoite challenge. In similar fashion, αβ T-cell clones with antiparasite activity have been generated after immunization with peptide antigens representing epitopes of the circumsporozoite protein of *Plasmodium yoelii* (Takita-Sonoda *et al.*, 1996).

Conditioned media as a source of growth factors

Concanavalin A-activated supernatant (ConA-sup) is a rich source of IL-2, but contains little IL-4 or IFN-γ; supernatant from secondary MLC (MLC-sup) contains high levels of IFN-γ; supernatant of EL-4 tumour cells (EL-4-sup) contains IL-2 but not IFN-γ; some lines produce IL-4 as well. EL-4-sup can usually be used instead of ConA-sup.

ConA-sup

1. Culture 1.25×10^6 cells ml^{-1} rat or mouse spleen cells 24–48 h in a humidified 37°C, 5% CO_2 incubator, in supplemented DMEM-5 medium (without 3-[*N*-morpholino]propanesulfonic acid (MOPS)), in the presence of 2.5 μg ml^{-1} ConA.
2. Collect culture supernatant and remove cells by centrifugation. Remove residual ConA by absorption with a slurry of Sephadex G-25 (0.2 g ml^{-1}) with α-methylmannoside.
3. Assay ConA-sup for IL-2 content (e.g. with the HT-2 assay). Store aliquots frozen at –70°C.

MLC-sup

1. Mix 2.5×10^7 C57BL/6-responding mouse spleen cells with an equal number of irradiated (2000 rad) DBA/2-stimulating mouse spleen cells in 20 ml supplemented DMEM-5 medium. Culture for 10–14 days at 37°C, 5% CO_2. (*Note*: This particular combination of mouse strains is most effective in generating MLC-sup.)
2. Collect cells from primary MLC in a 50-ml tube and wash twice with supplemented DMEM-5.
3. Prepare secondary MLC by mixing 6×10^6 primary MLC cells with 2.5×10^7 irradiated DBA/2-stimulating cells in 20 ml supplemented DMEM-5 medium. Culture for 36 h at 37°C, 5% CO_2.
4. Collect culture supernatant and process as for ConA (except for the absorption step).

EL-4-sup

1. Culture EL-4.IL-2 murine lymphoma cells (10^6 cells ml^{-1}) for 4 h in supplemented DMEM-10 medium and 20 ng ml^{-1} phorbol myristate acetate (PMA).
2. Collect cells and wash three times. Incubate for 36 h at 37°C, 5% CO_2.
3. Collect culture supernatant and process as for ConA-sup (omitting the absorption step).

T-cell Clones expressing TCR γδ

Preparing human γδ T-cell clones is not particularly problematic, but generating their murine counterparts has proved to be more difficult. At present only a few stable murine lines and clones are available. Murine γδ T-cell clones have been isolated and propagated under conditions avoiding possible competition with αβ T cells, presumably because αβ T cells adapt better to tissue culture and grow faster. The first reported murine clones were derived from congenitally thymus-deficient BALB/c nu/nu mice, after repeated immunization with B10.BR spleen cells (Matis *et al.*, 1987, 1989). The nearly complete absence of αβ T cells in the thymus-deficient mice allowed selective enrichment and cloning of the less thymus-dependent γδ T cells. The clones isolated in this and a similar study proved to be specific for major histocompatibility complex (MHC) encoded cell surface non-classical class I and class II molecules (Matis and Bluestone, 1991). They express Vγ4 in association with Vδ5, and TCR junctional differences appear to dictate ligand specificities (Rellahan *et al.*, 1991).

A natural tissue source of γδ T cells that lack contaminating αβ T cells is the murine epidermis (Nixon-Fulton *et al.*, 1986; Stingl *et al.*, 1987; Asarnow *et al.*, 1988). γδ T cells, also known as Thy-1^+ dendritic epidermal cells (DECs), are normally present in this tissue in rather large numbers, forming a loose network. In contrast, αβ T cells extravasate into the

epidermal layers only under pathological conditions. In partially enriched preparation of epidermal cells, DECs grow slowly and eventually form lines (Nixon-Fulton *et al.*, 1988). This process may be facilitated by the ability of DECs to recognize autologous keratinocytes (Havran *et al.*, 1991), which are still present in these cultures. The epidermal γδ T cells virtually all express Vγ5 in association with Vδ1 without junctional variations (Asarnow *et al.*, 1988). The molecular nature of their ligands remains unknown. Murine γδ T-cell clones have also been derived from malaria-immunized mice (Tsuji *et al.*, 1994). Here, TCR-β 'knockout' mice were used as a source of γδ T cells, thus eliminating competition from αβ T cells. These spleen-derived γδ T-cell clones express either Vγ1 in association with Vδ5, or Vγ7 together with Vδ4 (Tsuji *et al.*, 1996). One clone protected mice from challenge with *P. yoelii* sporozoites, and all responded to re-stimulation with extracts of parasitized erythrocytes and spleen cells. Nevertheless, the specific trigger for the response has not been identified.

To our knowledge, no clones have yet been generated that express either Vγ2 or Vγ6 γδ TCRs. Only a few researchers have been successful in cloning murine γδ T cells, so generally applicable rules have not yet been developed, and the procedures given below must be regarded as tentative. However, some of the peculiarities described, such as the extremely long incubation period prior to the appearance of γδ T-cell clones, should be taken into consideration when starting a cloning project involving murine γδ T cells. Detailed procedures for the isolation of DEC clones and for γδ T-cell clones derived from malaria-infected mice are given below.

DEC clones

Cell preparation

Several epidermal T-cell clones (Reardon *et al.*, 1995) have been produced using a method described by Nixon-Fulton *et al.* (1986), with some modifications. Sacrificed mice are washed with a liquid antibacterial soap to remove hair oils and rinsed with tap water. The wet intact trunk and ear skin is covered with a depilatory (e.g. Nair without aloe vera), massaged into the hair with a gloved finger, and incubated for 15 min, after which the hair is removed gently with a #22 scalpel. The intact skin is washed again with the soap to remove the depilatory and excess hair, and is rinsed with deionized water. While holding the animal by the tail, the skin is rinsed with 70% ethanol, and the mouse placed on ethanol treated paper towels on a cork or styrofoam board. After the limbs have been secured, the abdomen and chest skin is removed with sterilized scissors, cutting the skin over the suprapubic areas, along the flanks and across the neck area. Care must be taken not to cut into the peritoneal cavity. The skin then is peeled off and placed into a sterile dry Petri dish where it is rolled up with epidermis facing outwards to prevent desiccation of the dermal side. The animal is turned over, and the skin is cut across the caudal back and neck and again peeled from the fascia overlying muscle. The skin is placed into the Petri dish as before, while other animals are being

prepared. When all skin has been harvested, each piece of skin is turned over with the epidermal side down.

Using scissors, the fat and small vessels are teased and trimmed off the dermal side to reduce the possibility of contaminating the preparation with blood-derived T cells. The trimmed skin is placed into new dry Petri dishes with the dermal side facing down and left for about 15 min to adhere to the dish. Trypsin (0.3% in phosphate buffered saline (PBS)) containing glucose (0.1%) is gently placed into the dish until the epidermis is completely covered. Sterile metal screens or other like materials can be placed onto skin that has detached from the bottom to prevent it from floating. The skin is left in trypsin overnight at 4°C.

The next day, the skin is removed from the Petri dish and placed dermal side down into new dry Petri dishes. Using a #22 scalpel or forceps, the epidermis is very gently scraped from the dermis. This epidermal paste is placed in a 50-ml polystyrene tube, covered with 10 ml of the trypsin solution plus 0.1% DNase, and incubated at 37°C for 15 min, with gentle agitation of the tube to produce a single-cell suspension. An equal volume of balanced saline solution (BSS) containing 5% fetal bovine serum (FBS) is added to this slurry of cells and is then passed quickly through a small plug of sterile nylon wool in a 10-ml syringe to remove the clumps of stratum corneum. The cells that are eluted from the nylon wool are washed with BSS-FBS, and placed in a medium- to large-sized flask in complete medium for overnight incubation to remove adherent keratinocytes and fibroblasts.

The next day, the non-adherent cells are passed over a nylon-wool column and incubated for 30 min at 37°C for the cells to adhere before elution to remove non-T cells. (*Note*: The epidermal slurry can be incubated on the nylon wool columns directly, bypassing the overnight incubation.) Eluted cells are cultured in 24-well plates at 1×10^6 to 5×10^6 cells per well, in wells previously coated with 10 μg ml^{-1} pan-anti-γδ TCR monoclonal antibody, 403A10 (Itohara *et al.*, 1989) or GL-3 (Pharmingen), together with 10 U ml^{-1} recombinant IL-2 (R and D Systems, Minneapolis, MN, USA). Confluent wells are moved into six-well antibody-coated plates. Clonal T-cell lines are isolated by limiting dilution in 96-well microtitre plates by diluting an aliquot of cells to 10 cells ml^{-1} and adding 0.1 ml of this to each well of one or more 96-well microtitre plates. Although antibody coating is no longer necessary, 10 units ml^{-1} IL-2 must be added to the medium. Clones are expanded into 24-well plates and stained by flow cytometry to test for uniform TCR staining with 403A10 or GL-3 antibodies. The cells also need to be stained with the anti-Vγ5 (Vγ3 by other nomenclature) antibody F536 (Pharmingen), to ensure that the cells are of the epidermal type, since other cells derived from contaminating peripheral blood may have been selected.

γδ T-cell clones from *P. yoelii* sporozoite-immunized mice

Immunization of αβ T-cell-deficient mice with *P. yoelii* sporozoites has been described in detail elsewhere (Tsuji *et al.*, 1994, 1996). Briefly, mice

were immunized by bites of γ-irradiated, malaria-infected *Anopheles stephensi* mosquitoes (daily for 2 weeks). Five days after the last exposure to the moquitoes, spleens and livers were removed, and clones were generated by the following procedure.

Bulk cell culture

Responder cells from spleen and liver of immunized mice were cultured in the presence of feeder cells. To prepare feeders, normal spleen cell suspensions were depleted of erythrocytes by treatment with Tris-buffered 0.15 M ammonium chloride, washed twice, counted, and irradiated (3000 rad). Irradiated spleen cells (4×10^6) were added as feeders to each well of a 12-well tissue culture plate.

Bulk spleen cells and non-parenchymal liver cells from immunized mice were prepared in the same fashion, but omitting the irradiation. Responder cells (6×10^6) were added to each well, and the culture continued under standard conditions (37°C, 5% CO_2). Approximately half of the culture medium was exchanged every 2 days with fresh medium. One week after beginning the culture, T-cell blasts were purified with lympholyte-M (Cedarlane) and placed into new cultures (6×10^6 cells per well of a new 12-well plate). One week later, the cells were collected and counted.

Cloning

Cloning was carried out by limiting dilution. Cells (10, 10^2 and 10^3) were placed in each well of a 96-well tissue culture plate (one plate for each dilution), together with 10^6 irradiated and erythrocyte-depleted normal spleen cells in each well as feeders. Approximately half of the culture medium was replaced every 3 days. After 2 weeks, the feeder cells were replenished, continuing to replace half of the culture medium every 3 days. After an additional 2 weeks, the feeder cells were replenished again, and the culture continued with medium changes as before. After an additional 1–2 weeks, colonies of γδ T cells appeared. These were expanded once or twice more in the presence of normal spleen cell feeders, then transferred into 24-well plates at 10^5 cells per well. Cell culture was continued from here on by changing approximately half of the culture medium every 3 days, re-stimulating with splenic feeders every 2 weeks.

Culture medium

- RPMI 1640 plus sodium bicarbonate (2 g l^{-1})
- sodium pyruvate (100×)
- Modified Eagle's medium (MEM) non-essential amino acids (100×)
- MEM vitamins (100×)
- MEM essential amino acids (50×)
- L-glutamine (100×)
- 2-mercapto-ethanol (2ME) (5×10^{-5} M)

- penicillin/streptomycin (100×)
- gentamycin (10 μg ml^{-1})
- 10% FCS
- 2% EL4 supernatant (equivalent to 100 units of IL-2 per millilitre).

Important details

- Erythrocytes must be lysed.
- The medium used for the initial bulk cultures should contain twice the concentration of EL4 supernatant compared to the medium used for expansion of αβ T-cell clones.
- γδ T cells grow much slower than αβ T cells. Therefore, it usually takes 6–8 weeks or more for γδ T-cell colonies to become visible after cloning.
- After the number of cloned γδ T cells reaches more than 10^5 cells per well (24-well plate), purification of the γδ T cells one week after each stimulation may accelerate the growth of these cells.
- Unlike αβ T cells, the morphology of γδ T-cell clones is quite heterogeneous, with large differences in cell sizes and shapes.

Transfer Studies using Cloned T Cells

Like normal T lymphocytes, cloned T cells can be used for adoptive transfer studies. In contrast, T-cell hybridomas tend to behave like lymphoid tumours, having lost many of their effector functions. Numerous transfer experiments have been carried out with αβ T-cell lines and clones. Such clones are capable of completely changing the outcome of an immune response. $CD8^+$ T cells specific for a single nonameric peptide epitope of a bacterial protein provided protection against bacterial infection in a mouse model (Harty and Bevan, 1992). Transfer of patient-derived, *in vitro* antigen-stimulated and expanded T-cell clones is under development for the treatment of both cancer and chronic autoimmune diseases. However, a detailed description of such studies and experimental systems is beyond the scope of this chapter. Below, two examples are given to illustrate the procedure of clonal transfer using both αβ and γδ T cells, and the possible outcome of such experimentation.

Host protection by *Listeria*-specific CD8+ T-cell lines

$CD8^+$ T-cell lines (Harty and Bevan, 1992) were derived from DBA/2 mice after intravenous infection with a sublethal dose of *Listeria monocytogenes* strain 43251 (5×10^2 colony-forming units (cfu)), 7 days before spleen harvest and *in vitro* stimulation. For primary *in vitro* stimulation, 35×10^6 to 40 $\times 10^6$ immune spleen cells were cultured for 7 days with 3×10^6 target cells (irradiated PHem3.3, a P815 mastocytoma (DBA/2, $H\text{-}2^d$, MHC class II negative) derived transfectoma expressing bacterial listeriolysin O (LLO)). Re-stimulations were carried out weekly using irradiated PHem3.3 in the presence of 5% supernatant from ConA-stimulated rat splenocytes.

$CD8^+$, LLO-specific T-cell lines derived from these cultures were then used in adoptive transfer experiments. $CD8^+$ T cells (1.5×10^7), derived from the LLO-specific line 479-2, when injected intranvenously just prior to infection with >1 LD_{50} of *L. monocytogenes* reduced the bacterial load by >3 $\log_{10}$ in spleens and by >1.5 $\log_{10}$ in livers, as compared to control mice. The protective ability of these cells was dose dependent and specific.

Another $CD8^+$ T-cell line, derived from mice immunized with the LLO peptide 91-99 (line 603-1-2), also reduced bacterial titres *in vivo* by >1 $\log_{10}$, even when administered as late as 24 h after bacterial infection.

Neutralization of IFN-γ *in vivo* exacerbates listeriosis, and treatment of mice with IFN-γ prior to infection results in partial protection against this bacterium. However, *in vivo* protection by the listeria-specific T-cell line, 479-2, was not inhibited by neutralizing with anti-IFN-γ monoclonal antibodies (mAbs), suggesting that protection by these cells is independent of IFN-γ (Harty *et al.*, 1992).

T-cell lines specific for another listeria protein, p60 (a secreted protein involved in bacterial septation and implicated in infection of non-phagocytic cells), were also found to be protective (Harty and Pamer, 1995). In this study, cells were harvested at day 8 or 9 after re-stimulation, washed and injected intravenously (5×10^6 to 8×10^6 in 0.2 ml PBS, 1 h prior to intravenous injection of 1×10^5 *L. monocytogenes*). *In vivo* protection as measured by the number of colony forming units per organ was determined 72 h after infection.

Host protection by a γδ T-cell clone

γδ T-cell clones were derived from *P. yoelii* -immunized TCR-α 'knockout' mice (described in detail above). One of these clones, 291-H4, inhibited the development of liver stages of *P. yoelii*, following sporozoite inoculation (Tsuji *et al.*, 1994, 1996).

For adoptive transfers, γδ T-cell clones were harvested 10–14 days after re-stimulation. Cells (10^7) were washed and re-suspended in RPMI 1640 medium containing recombinant human IL-2 (2000 units ml^{-1}), at a concentration of 2×10^7 cells ml^{-1}. Cell suspension (0.5 ml) was injected intravenously into each mouse. Mice were then challenged with 3×10^5 sporozoites of *P. yoelii*, 4 h after the cell transfer.

Parasite development, when using clone 291-H4, assessed 42 h after infection, was partially inhibited (in four independent experiments inhibition varied between 46% and 67%), whereas other clones were not inhibitory. The mechanism of inhibition remains unclear, but is probably different from the protection seen with αβ T cells, because the protective clone 291-H4 does not exhibit specificity for the pathogen.

◆◆◆◆◆◆ T-CELL HYBRIDOMAS

Fusion Lines

The basic technique of immortalizing lymphocytes by fusion to tumour cells was introduced about 20 years ago (Köhler and Milstein, 1975a; Taniguchi and Miller, 1978; Kappler *et al.*, 1981). The technique requires selectable fusion lines, efficient fusion agents and reliable selection/cloning protocols.

Although practically all cells can be forced to fuse with other cells, only some are suitable for hybridoma generation (Shulman *et al.*, 1978; Galfré *et al.*, 1979; Kearney *et al.*, 1979). In fact, identifying a good fusion line is no small undertaking, as can be appreciated by the fact that there is still no efficient fusion line available for the generation of human T-cell hybridomas, despite considerable efforts to identify such cells.

Fusion lines should be tumour cells that are capable of unlimited growth in tissue culture, able to form stable hybridomas after fusion with normal cells, and selectable. Among several selectable genetic markers, irreversible deficiency in the enzyme hypoxanthine–guanine phosphoribosyl transferase (HGPRT) has proved to be the most reliable, and it is currently the most widely used marker (Schreier *et al.*, 1980). DNA synthesis occurs essentially via two pathways: a main pathway of *de novo* synthesis, and an alternative or rescue pathway reutilizing DNA breakdown products. The rescue pathway depends on the enzyme HGPRT, which uses hypoxanthine as a precursor in the synthesis of purine bases. Selection is accomplished by blocking the *de novo* synthetic pathway with the drug aminopterin. Because the rescue pathway is also lacking, HGPRT-deficient fusion lines cannot grow in the presence of aminopterin. Being unable to synthesize DNA, they rapidly die unless they are rescued by fusion to an HGPRT-positive cell, the normal fusion partner. (Because they do not divide without specific stimulation, unfused normal cells will also die eventually, although more slowly.) HGPRT-deficient cell lines can be obtained by selection for resistance to the purine analogue 8-azaguanine. This drug is lethal to cells incorporating it as a precursor in the alternative pathway, despite ongoing DNA synthesis via the main pathway. Cells surviving this treatment, therefore, are typically HGPRT-deficient mutants. This selection is very efficient and has worked very well with both B- and T-cell tumours. Revertants are not normally observed. A similar selection procedure has been used to generate cell lines defective for the enzyme thymidine kinase (TK), which can overcome the aminopterin block of the main pathway by using thymidine (Schreier *et al.*, 1980). In this case, enzyme loss variants are selected for resistance to the pyrimidine analogue 5'-bromodeoxyuridine (BrdU).

The survival of a new hybridoma depends on its ability to switch to the alternative pathway of DNA synthesis shortly after the fusion. To assist with this transition, tissue culture medium must be supplemented with precursors of the alternative pathways, hypoxanthine and thymidine. Therefore, the selection medium for $HGPRT^-$ or TK^- fusion lines is known as HAT (hypoxanthine, aminopterin, thymidine) medium.

For B-cell fusions, appropriate myeloma fusion lines were available by 1979, including Ig-negative mouse myeloma lines that could be used to generate B-cell hybridomas exclusively producing antibodies derived from the normal cell partner (Shulman *et al.*, 1978; Galfré *et al.*, 1979; Kearney *et al.*, 1979). T-cell fusion lines were developed with some delay, concomitant with the somewhat slower progress of T-cell research. Although several T-cell tumours have been tried, by far the most widely used tumour partner cell in mouse T-cell fusions became the AKR strain-derived thymus lymphoma BW5147, first described by Hyman and Stallings (1974). HGPRT-negative variants of this cell line were used to generate the first antigen-specific mouse T-cell hybridomas (Kappler *et al.*, 1981). However, this thymus-derived lymphoma contains its own functionally rearranged TCR-α and TCR-β genes (Letourneur and Malissen, 1989; White *et al.*, 1989), which are not expressed on the cell surface because of a defect in CD3 complex protein genes. Upon fusion with a normal, $CD3^+$ T lymphocyte, the tumour-derived TCR-α and TCR-β chains are expressed on the hybridoma cell surface along with those of the normal cell. Thus, early T-cell hybridomas could express, together with the normal cell-derived TCR, up to four different TCR αβ combinations, or even more if one takes into account the fact that allelic exclusion at the α gene locus is not absolute. Obviously, the expression of multiple TCRs on a single hybridoma was a problem in studies concerned with ligand specificities. In fact, a BW5147-derived contribution to specificities for $I\text{-}A^b$ has been identified (Yeh *et al.*, 1984). However, the multiple combinations of TCR chains in such hybridomas provided an opportunity to test the role of individual TCR chains in determining specificity. In particular, the specificities of unselected, *in vitro* created pairs of TCR chains could be examined.

To eliminate the problem of endogenous TCR gene expression in BW5147, TCR gene loss variants were generated, lacking either functional TCR-α rearrangements (BW/α^-), or both TCR-α and functional TCR-β gene rearrangements (BW $\alpha^-\beta^-$) (White *et al.*, 1989). BW $\alpha^-\beta^-$ still contains non-functional TCR gene rearrangements for both α and β that give rise to partial gene transcripts, but they no longer contribute to surface-expressed TCR protein.

BW $\alpha^-\beta^-$ has been modified for certain experimental requirements. For example, the fusion line does not express CD8, and it tends to suppress CD8 expression in hybridomas generated with $CD8^+$ cells, whereas CD4 expression is not inhibited (Carbone *et al.*, 1988). This has seriously hampered specificity studies with MHC class I-restricted T cells, which typically are CD8 dependent. To overcome this problem, a $CD8^+$ variant of BW $\alpha^-\beta^-$ has been generated by gene transfection (Burgert *et al.*, 1989). Briefly, a vector containing the gene encoding murine CD8α (pSFSVn-Ly2α, provided by Dr J. Parnes, Stanford University, CA, USA) was linearized and transfected into BW $\alpha^-\beta^-$ by electroporation. $CD8^+$ variants were identified cytofluorimetrically. One of these, expressing high cell surface levels of CD8, was chosen as a novel fusion line (BW Lyt2-4). It appears that, in contrast to natural CD8, BW $\alpha^-\beta^-$ is not able to downregulate expression of the transgenic CD8α gene. Because the isolation of BW

Lyt2-4 depended on its acquisition of G418 resistance (pSFSVn-Ly2α contains the neogene as a selectable marker), G418 selection must be maintained during the generation of hybridomas with this fusion line (see below).

T-cell hybridomas are often used to measure the initial antigen recognition event, i.e. the interaction between TCR and Ag/MHC complexes that leads to T-cell activation. Conventional methods for measuring this activation use bulk assays that yield an average read-out of T-cell activation (see below). This greatly limits sensitivity in detecting antigen recognition, and responses of individual cells will go unnoticed. To overcome this limitation, Karttunnen *et al.* (1992) and Sanderson and Shastri (1993) have developed the *'lacZ* assay'. Briefly, based on the finding that the 'nuclear factor in activated T cells' (NFAT) DNA element within the IL-2 gene enhancer controls transcriptional regulation of the IL-2 gene, a transfectable construct was developed in which the *Escherichia coli* β-galactosidase (*lacZ*) gene was placed under the control of NFAT. In transfectants, this leads to an increase in *Esch. coli* β-galactosidase activity when these cells are activated to produce IL-2. Unlike secreted IL-2, β-galactosidase remains sequestered within activated cells. β-Galactosidase activity can be measured with chromogenic or fluorogenic substrates (see below), thus allowing detection of activated T cells. Using the chromogenic substrate and light microscopy, individual activated T cells can be distinguished by their blue colour. Initially, the *lacZ* T-cell activation assay was used for the detection of rare antigen presenting cells. More recently, two *lacZ* inducible fusion partners have been derived by transfecting BW $\alpha^-\beta^-$ with the NFAT-*lacZ* DNA construct (Sanderson and Shastri, 1993). Fusion of normal T cells with the *lacZ*-inducible cell lines allows for automatic generation of *lacZ*-inducible antigen-specific T-cell hybridomas. Measurement of T-cell antigen responses with the *lacZ* assays is faster, more sensitive and less expensive than conventional IL-2 assays (see below), and makes it possible to detect activation in single T cells, or rare ligand binding APCs. Application of this cloning strategy may enable the identification of unknown T-cell antigens involved both in pathological and normal immune responses.

To generate *lacZ*-inducible fusion partners, BW $\alpha^-\beta^-$ cells were transfected with a linearized NFAT-*lacZ* DNA construct, and selected in medium containing 400 U ml^{-1} hygromycin. Because BW $\alpha^-\beta^-$ does not express a TCR, transfectants were selected based on their ability to express *lacZ* activity after incubation with the mitogen PMA/ionomycin, which stimulates NFAT transcriptional activity independently of the TCR. Potential fusion partners were first selected based on high *lacZ* expression, using a fluorogenic *lacZ* substrate and cytofluorimetry (see below). Second, a small collection of high *lacZ* expressors was tested for their ability to give rise to *lacZ*-inducible T-cell hybrids. The transfectant BWZ.36 was chosen as the optimal fusion partner, by measuring *lacZ* induction after CD3 cross-linking. (*Note*: Hybridoma selection with the BWZ.36 fusion partner requires culture in the presence of both aminopterin and hygromycin.) To allow this fusion partner to be used for immortalization of MHC class I restricted and allospecific T cells, the line was next transfected with a CD8α retrovirus,

using a Lyt2a construct. Transfectants were selected in medium containing G418, and analysed cytofluorimetrically for surface expression of CD8α. One transfectant (BWZ.36/CD8α), which expressed CD8 at levels comparable to those of a normal CTL clone, was found to be an efficient fusion partner for alloreactive and other MHC class I-specific T cells (Sanderson and Shastri, 1993).

While BWZ.36 and BWZ.36/CD8α successfully circumvent the limitations of bulk assays for T-cell activation (see below), they do not allow the isolation and cloning of the antigen-reactive cells because these cells are destroyed by the detection method (see below). An assay for detecting activation at the single-cell level that does not result in cell death would therefore be much more desirable. Using the gene encoding green fluorescent protein (GFP), a protein originally isolated from the jellyfish *Aequorea victoria* and fluorescent upon excitation with violet or blue-green light, this may indeed be possible (Anderson *et al.*, 1996). Engineered GFP variants adapted to the requirements of cytofluorometry have already been found useful as reporter genes when measuring murine cell activation. With a GFP-transfected fusion line it should thus be possible to isolate antigen-reactive hybridomas from mixed cell populations, by sorting for fluorescence-activated cells.

BW $\alpha^-\beta^-$, the most widely used T-cell fusion line for the generation of mouse T-cell hybridomas, has also been used for the generation of rat T-cell hybridomas, but it does not appear to be suitable for generating stable hybridomas with human T cells.

A γδ T-cell tumour suitable for generating hybridomas with γδ T cells has not been identified, but BW α^- and BW $\alpha^-\beta^-$, in contrast to the original BW5147, can be used to generate hybridomas expressing γδ TCRs (Born *et al.*, 1987). Although BW $\alpha^-\beta^-$ still contains its own endogenous TCR-γ gene rearrangements, they are non-functional and are not expressed on the cell surface. The γδ T-cell hybridomas generated with BW $\alpha^-\beta^-$, comparable in stability to αβ T-cell hybridomas, were used to isolate γδ TCRs and to obtain partial protein sequences of surface-expressed TCR-δ (Born *et al.*, 1987). Some of the first studies reporting γδ T-cell responses to mycobacterial antigens utilized γδ T-cell hybridomas (Happ *et al.*, 1989; O'Brien *et al.*, 1989). It should be noted, however, that hybridomas generated by fusions of γδ T cells with BW $\alpha^-\beta^-$ are heterohybrids, probably combining properties of αβ T cells with those of γδ T cells.

Hybridization

A number of agents promoting the fusion of cell membranes have been examined for cellular hybridization. Currently, the most widely used fusion agent for T and B lymphocytes is polyethylene glycol (PEG) (Galfré *et al.*, 1977). Briefly, mixtures of cells to be fused are incubated with dissolved PEG, washed to remove the fusion agent, allowed to recover overnight, and selected for acquisition of drug resistance starting the next day (see below for specific hybridization protocols). Typically, only a small fraction of the mixed cells undergo membrane fusions, and not all of these proceed to nuclear fusions required to form selectable hybrids.

Newly formed hybrids remain unstable for some time. Starting with two sets of chromosomes, they begin to eliminate a portion of them in a more or less random fashion. In fusions with normal human cells, human chromosomes seem to be lost selectively. This process will stop eventually, without a predictable end-point. Different hybridomas thus end up with *'n'* plus a variable number of additional chromosomes. Typically, hybridomas need to be selected several times before they become relatively stable, and even then, markers of interest may be rapidly lost if they are not required for the survival of the cells. Even after repeated subcloning, hybridomas tend to be less stable than clones of normal cells, making it necessary to maintain frozen stock of early passages and to keep periods of continued cell culture as short as possible.

A protocol for the hybridization of murine T cells with BW $\alpha^-\beta^-$ is given below. In modified form, this protocol is also suitable for use with any of the variants of this fusion line.

Hybridization, hybridoma cloning and hybridoma maintenance

Tissue culture medium

Most of the media currently used for the long-term culture of mouse lymphocytes seem to be adequate. We prefer Iscove's modification of Dulbecco's medium.

Polyethylene glycol

PEG 1540 (Sigma) is stored in 10-g aliquots (approximately 10 ml) in 50-ml polypropylene tubes. Prior to each use, PEG is boiled for exactly 10 min (in boiling water to avoid overheating), and the melted PEG is thorougly mixed with 10 ml of culture medium (without supplements). Fifty percent of the PEG solution (10 ml) is then filtered through a 0.22-m Nalgene sterile filter and discarded (as a wash). The next 10 ml is passed through the same filter and kept at 37°C until use. The pH of the 50% PEG solution should be around 7.2. This relatively complicated procedure of preparing the 50% PEG solution was introduced in the early 1980s, with highly variable PEG 1540 lots and sterile filters that contained detergent. It is now possible to buy at a higher cost PEG lots prescreened for fusion efficiency, and it may no longer be necessary to prewash the filters with warm PEG with the advent of detergent-free filters. However, we still include this step.

Cells

For T-cell fusion experiments, we use approximately 2×10^7 fusion line cells, although the number of normal cells may vary. We have successfully carried out fetal thymocyte fusions with as few as 10^5 thymocytes, with a yield of 0–20 hybridomas per fusion. Large numbers of hybridomas (up to several thousand) are obtained with activated T cells (purified T-cell blasts) in much larger numbers (up to 10^8 per fusion). Therefore, the number of clones generated may vary greatly.

Fusion

1. Thorougly wash 2×10^7 BW$\alpha^-\beta^-$ (or derivative) cells, as well as the normal fusion partners, using serum-free BSS. Then combine the cells in a 50-ml conical polypropylene tube and wash once more. Suction off the supernatant, spin again for a short period of time (3 min or so), and remove all liquid supernatant.
2. Break up the wet cell pellet by gently tapping the tip of the tube, and then place the tube in a beaker containing dH_2O at 37°C in order to keep the pellet warm during the fusion.
3. Add to the pellet 1 ml of warm (37°C) 50% PEG solution, dropwise over a period of 45 s. While adding the PEG, continuously turn the tube to ensure equal distribution of the fusion agent. Incubate the tube for an additional 45 s at 37°C in the water beaker. After a total of 90 s in 50% PEG, slowly and gradually dilute PEG with culture medium. We recommend the following method (adapted from John Kappler). Add 1 ml of culture medium (without supplements or serum) dropwise over 30 s, then 2 ml over the next 30 s, then 3 ml, then 4 ml, then, after a total time of 2 min, add 40 ml of culture medium, close the tube and incubate for an additional 5 min at 37°C in a waterbath. During the dilution procedure it is necessary to gently turn or shake the tube to ensure that the 50% PEG solution is actually diluted further. The pellet should stay more or less intact to allow cell–cell contact in the presence of PEG for a prolonged period of time.
4. Spin at normal cell speed (approximately 200*g*) for 5 min at 37°C or room temperature, remove the supernatant, add 50 ml BSS (if possible without dislodging the pellet), and wash twice more. This is necessary to remove as much as possible of the toxic fusion agent.
5. After the last wash, add 10 ml of culture medium containing all usual supplements and serum, and gently break up the pellet using the same pipette. Add 20–40 ml of additional culture medium, depending on the subsequent cloning step (see below).

After this, the newly formed hybrids can be maintained in bulk culture or immediately cloned. For most purposes, an immediate cloning step is advisable and should be carried out before the actual selection begins.

Cloning

Using 96-well microtitre plates for tissue culture, we distribute the newly formed hybrids in 100-µl aliquots per well over several plates, depending on the anticipated number of selectable hybridomas. For example, for 100 hybridomas, four or five plates are considered adequate. With less than 37% growth-positive wells, the Poisson distribution predicts that more than 95% are derived from single cells in each well, i.e. clonal hybridomas. Obviously, the choice of an appropriate number of plates requires some experience. In general, it is better to anticipate a larger number of hybrids

because it becomes very difficult to deal with fusion experiments with a large predicted percentage of non-clonal hybridomas. Depending upon the purpose of the experiment, it might be possible to recover from such a problem by selecting a small number of potentially non-clonal cell lines and to subclone each one of them.

Selection

Hybridomas are selected using HAT. At 24 h after cloning, 50 µl HAT (diluted in culture medium with all supplements to make a 3× solution) is added to each well. Four days later, the medium must be changed, using 1× HAT. HAT-selectable hybridomas tend to begin to appear after 5–6 days of culture, although some hybrids seem to require as long as 20 days before they start to grow. The growing hybrids, filling part of the well bottom (5×10^4 cells or more) can be picked and transferred into 24-well plates. At this point, the culture medium used should no longer contain aminopterin but should be supplemented with thymidine and hypoxanthine (HT medium) at the same concentrations as before, for the subsequent two passages (regardless of how fast the cells grow). This is necessary because residual aminopterin can poison the new hybridomas if precursors of the alternative pathway of DNA synthesis are not provided in sufficient quantities. Only after at least two passages with HT can the new hybridomas be weaned into regular culture medium. If at this point many of the hybridomas die, weaning was probably begun too early and the cells should be transferred back into HT medium for several additional passages.

Repertoire Studies and the Problem of Representation

The primary purpose for generating hybridomas has been to immortalize and multiply individual B or T lymphocytes with properties of particular interest. However, cellular hybridization can also be used as a means of obtaining 'snapshots' of mixed cell populations, in developmental studies or when examining lymphocyte subset compositions during an immune response. For example, the sequence of TCR gene rearrangements during thymic maturation of T lymphocytes (both αβ and γδ) has been analysed using collections of thymocyte hybridomas representing subsequent stages of thymocyte development (Born *et al.*, 1985, 1986; Haars *et al.*, 1986). Incidentally, one such developmental study led to the discovery of the gene locus for TCR-δ, and the peculiarly interspersed organization of TCR-α and TCR-δ genes (Chien *et al.*, 1987). Probably of lesser importance, it has also been documented that T cells partially rearrange immunoglobulin genes in a developmentally ordered fashion (Born *et al.*, 1988). Developmental patterns found in these hybridoma studies are reasonably well correlated with the findings of other studies based on antibody staining or the analysis of mRNA derived from bulk T-cell preparations (Raulet *et al.*, 1985; Snodgrass *et al.*, 1985). However, it cannot be assumed that this will always be the case. Hybridoma formation is

clearly biased towards activated cells, so that within mixed cell populations such cells will be overrepresented after hybridization. It also seems to be true that closely related cell types are more likely to form stable hybridomas, suggesting that the fusion line BW5147 probably favours certain types of αβ T cells. Finally, as already discussed above, some of the properties of the normal fusion partner may be suppressed after hybridization, leading to a distorted phenotype, even when the fusion was unbiased.

Functional Competence

Although T-cell hybridomas have been used extensively to examine properties and functions of the T-cell receptor and other cell-surface molecules, as well as to study signalling pathways and consequences of signalling such as T-cell anergy and apoptosis (Evavold *et al.*, 1994; Brunner *et al.*, 1995), there is little evidence that they retain the effector functions of the normal cells from which they are derived (Haas *et al.*, 1995; Gu and Gottlieb, 1992; Gorczynski *et al.*, 1996). With few exceptions, hybridomas cannot substitute for freshly isolated cells or clones in adoptive transfer experiments, since they typically lack cytolytic abilities (but see the exceptions referred to above) and do not exhibit clear Th1/Th2 phenotypes in terms of cytokine production.

Hybridization of γδ T cells is still carried out with the same fusion partner that is used for αβ T cells, BW $\alpha^-\beta^-$. γδ T-cell hybridomas are therefore likely to exhibit a mixed phenotype, combining properties of γδ and αβ T cells (O'Brien *et al.*, 1989).

◆◆◆◆◆◆ T-CELL TRANSFECTOMAS

Retroviral gene transfer is gaining popularity as a means of introducing foreign genes into many cell types, including haematopoietic and primary cells (Miller *et al.*, 1993). Recombinant retroviruses contain both selectable markers and strong promoters, such as the cytomegalovirus (CMV) or long terminal repeat (LTR), which drive the transcription of the desired gene. Cells previously transfected with genes for the retroviral structural proteins *gag*, *pol* and *env* (packaging cell lines such as GP + E-86) can provide these in *trans* when a retroviral vector is introduced, so that they produce replication-defective virions continuously. Retroviral vectors commonly used have been described by Miller *et al.* (1993).

The murine stem cell virus vector (MSCV, versions 2.1 and 2.2), which contains the murine stem cell virus LTR promoter/enhancer, works well for infection of γδ T-cell hybridomas (Hawley *et al.*, 1992). High viral titres ($> 10^5$ cfu ml^{-1}), made by first transfecting the packaging cell with the retroviral plasmid then infecting naive packaging cells with viral supernatant harvested from the transfected cells, are needed to transduce dividing recipient cells efficiently. Stable transfectants result when the

viral sequences integrate into the target DNA. Precise integration of the retroviral vector is also possible, making this method useful for genetic studies (Miller *et al.*, 1993). One disadvantage of the technique is the size limitation of the gene that may be inserted in the retroviral vector (approximately 2 kb), limiting transduction to small genes or cDNAs.

The technique described by Miller *et al.* (1993) has been adapted for gene expression in T-cell hybridoma recipients.

Preparation of Viral Supernatants

Calcium phosphate transfection of retrovirus-packaging cells

The most critical component of calcium phosphate transfection is the pH of the transfection buffer, a *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (HEPES)-buffered saline solution, the optimum pH of which is between 7.05 and 7.12. Most protocols recommend making this reagent freshly (Graham and van der Eb, 1973), although we have achieved good results using frozen aliquots stored at –80°C. The selection system utilizing the bacterial *neo* gene (encoding aminoglycoside 3'-phosphotransferase), which confers resistance to G418, is convenient, but the sensitivity of cells to this drug varies greatly. The levels recommended here for T-cell hybridomas are higher than have been used for fibroblasts. A non-transfected control plate is always examined to ensure that positive selection is due to gene integration and expression, rather than cell resistance to the drug. We have used supplemented Iscove's modified Dulbecco's medium for all cell culture under standard conditions (37°C, 5% CO_2), but the exact medium is probably unimportant.

Materials

- Retrovirus packaging cells (GP + E-86 cells are recommended)
- retroviral vector plasmid DNA (neoresistant) containing the gene of interest (CsCl purified or equivalent)
- 5 M NH_4OAc/ETOH (1:5) for DNA precipitation
- 100 mg ml^{-1} G418 in BSS
- trypsin (0.3% solution in PBS)
- 1× G418 medium (culture medium with 1 mg ml^{-1} G418)
- 2 M $CaCl_2$
- HEPES-buffered saline (HBS)
- Coomassie Brilliant Blue stain/fix (CBB).

Procedure

1. Harvest retrovirus packaging cells, using 0.3% trypsin to detach cells from the tissue culture plate, by removing culture medium and covering cells with a small volume of trypsin solution for a few minutes at 37°C. Pipette to obtain a single-cell suspension. Dilute 10-fold in media, spin cells and discard supernatant. Plate the cells at

5×10^5 cells per 6-cm tissue culture dish, in 4 ml culture medium per plate. Prepare one extra plate for a mock transfection (one plate needed per DNA). Incubate overnight.

2. Precipitate 10 µg of DNA with 5 volumes of NH_4OAc/ETOH to sterilize. Redissolve the DNA in 175 µl of sterile *dd*H_2O. Add 25 µl of sterile 2 M $CaCl_2$ to the DNA. Set up a tube with sterile water and $CaCl_2$ for the mock transfection.
3. Place 200 µl of freshly thawed HBS into a 1.7-ml Eppendorf tube. Add the DNA/$CaCl_2$ mixture dropwise to the HBS while gently vortexing the tube. Repeat with the water/$CaCl_2$ tube. Allow the tube to sit for 30 min at room temperature. A fine precipitate should form (DNA and calcium phosphate co-precipitate).
4. Replace the medium on the retrovirus packaging cells with fresh medium. Add the DNA precipitate to the medium dropwise, while gently swirling the plates. Incubate cells for 16–20 h, and replace medium. Incubate for a further 24 h.
5. Place culture supernatant in 15-ml conical tubes and spin at 3000*g* for 5 min at 4°C to remove all cells and cell debris. This low titre viral transient transfection supernatant may be used to infect naive packaging cells, but this titre is generally too low to be useful.
6. Change 1× G418 medium every 3–4 days. By day 6, a difference between the real and mock transfected plates should be obvious. When a dilution becomes confluent, replace the G418 medium with plain medium. Culture for 2–3 days more, place the supernatant in a 15-ml conical tube, and spin at 3000 rpm to remove debris. This intermediate titre viral supernatant may be stored at –70°C or used immediately for viral infection of naive packaging cells. A good transfection will produce as many as several thousand colonies, but only one is really needed to proceed.

Viral infection of retrovirus-packaging cells

For infection and integration of the desired gene into T-cell hybridomas, the viral titre needs to be high. Although high titres may be obtained from the primary fibroblast transfectants, retroviral-producing fibroblasts generated by infection may produce higher titres. We describe here how these can be produced. We recommend that you proceed with this step, but titre primary virus at the same time (see next section), so that in any case you will be ready for T-cell transfection. Here, the polycationic compound polybrene is used to increase the efficiency of infection of both the packaging cell and the gene-targeted cell, neutralizing the negative charges present on the cell surface so that virus and cells do not repel one another (Miller *et al.*, 1993). Tunicamycin, an inhibitor of glycosylation, is used to prevent viral envelope glycoproteins produced by the packaging cell line from blocking the normal receptors for the retrovirus which would otherwise occur (Rein *et al.*, 1982). The need for tunicamycin can be overcome, if desired, by switching packaging lines.

Materials

- Retrovirus packaging cells (GP + E-86 cells)
- supernatant from $CaPO_4$ transfected viral packaging cells
- 100 mg ml^{-1} G418 in BSS, 1× G418 (1 mg/ml^{-1} G418 in medium),
- trypsin (0.3% solution)
- 100 µg ml^{-1} tunicamycin (in sterile *dd*H_2O)
- 400 µg ml^{-1} polybrene (in BSS, sterile filtered).

Procedure

1. Seed four 10-cm tissue culture dishes with 2×10^5 packaging cells on 10 ml culture medium. Incubate overnight. (*Note*: Tunicamycin is rather toxic, and a decrease in cell viability will probably be evident. It may be necessary to experiment with the dose of this drug if too many cells (>75%) die.)
2. Add 10 µl of 100 µg ml^{-1} tunicamycin to each plate and incubate overnight.
3. Replace medium. Add 100 µl of 400 µg ml^{-1} polybrene to each plate and swirl gently. Add 1 ml, 0.1 ml and 10 µl viral supernatant from the $CaPO_4$ transfection to each of three plates. If the viral titre is expected to be high, add two more dilutions. The fourth plate will serve as the uninfected control. Incubate plates for 16–24 h before replacing the media with 1 mg ml^{-1} G418 medium. Medium should be changed every 2–4 days.
4. Colonies should be established in about 9 days and the non-infected cells will have died off. Colonies can be picked if desired, but, if not, when cells are confluent, replace the G418 medium with plain medium and culture for 2–3 days. Place the supernatant in a 15-ml conical vial and spin at 3000*g* for 5 min to remove all cell debris. Supernatant can be kept at –70°C until needed, preferably in aliquots to avoid repeated freezing and thawing.

Determining the viral titre in the supernatant

Supernatants with titres of $\geq 10^5$ cfu ml^{-1} are required for efficient infection of T-cell hybridomas. Determination of viral titres takes approximately 10 days, and repeated freezing and thawing of the viral supernatant can decrease the titre of the virus.

Materials

- NIH-3T3 or HeLa cells
- supernatant from virally infected packaging cells
- 100 mg ml^{-1} G418 in BSS
- 1× G418 medium (1 mg ml^{-1} G418 in medium)
- trypsin (0.3%)
- 100 µg ml^{-1} tunicamycin (in sterile *dd*H_2O)
- 400 µg ml^{-1} polybrene (in BSS, sterile filtered)
- Coomassie Brilliant Blue stain/fix (CBB).

Procedure

1. Plate six 6-cm tissue culture dishes each with 1×10^5 NIH-3T3 cells in 4 ml medium. Incubate overnight. Five plates will be used to dilute the viral titre, and the last plate as a non-infected control.
2. Add 40 ml of 400 μg ml^{-1} polybrene to each plate. Remove 0.4 ml medium from the first plate and discard. Add 0.4 ml of viral supernatant to this plate, swirl and serially transfer 0.4 ml medium to the next plate. Repeat this for the next three plates. Discard the last 0.4 ml medium. Incubate plates for 16–20 h, and then replace the medium with 1× G418 medium.
3. Change the G418 medium every 3–4 days until the colonies are mature. This normally takes 9–10 days.
4. To calculate the number of cfus per millilitre, visualize the colonies by washing with 2 ml BSS and staining with Coomassie Blue. (Add 2 ml CBB and swirl the plates until desired blueness is attained, then remove the stain.)

Viral Infection of T-Cell Hybridomas with Retrovirus-containing Supernatants

Once a high-titre viral supernatant has been made, infection of the target cell can proceed. A number of amphotrophic viruses use the sodium dependent phosphate symporters as cell surface retroviral receptors (Miller and Miller, 1994), and for this reason target cells are first incubated in phosphate free medium to upregulate the phosphate receptors. This is particularly important for T-cell hybridomas, which are difficult to infect compared to other cell types.

Materials

- Exponentially growing T-cell hybridoma recipient
- high titre viral supernatant from packaging cells (>10^5 cfu ml^{-1})
- RPMI medium 1640, sodium phosphate free (Gibco catalogue No. 11877-024)
- 100 mg ml^{-1} G418 in BSS
- 2× G418 medium (2 mg ml^{-1} G418 in medium)
- 100 μg ml^{-1} tunicamycin (in sterile water)
- 400 μg ml^{-1} polybrene (in BSS, sterile filtered).

Reagents

- HEPES buffered saline (HBS), 50 ml: 5 ml 0.5 M HEPES, pH 7.1 (pH is critical), 6.25 ml 2 M NaCl, 0.5 ml 150 mM $NaPO_4$, pH 7.0 (pH is critical), 38.25 ml *dd*H_2O (sterile); divide stock into 1-ml aliquots and store at –70°C, or prepare fresh each time.

- Commassie Brilliant Blue stain/fix, 1 l: 0.35 g Coomassie Brilliant Blue, 454 ml methanol, 92 ml glacial acetic acid, 454 ml dH_2O; sterile filter.

Procedure

1. Culture 2×10^6 T-cell hybridoma recipient cells in 10 ml RPMI (phosphate free) medium for 16–20 h. (*Note*: TCR loss variants of hybridomas often are generated spontaneously in culture, and can be isolated by cloning. This is useful if TCR genes are to be transfected.)
2. Transfer 1×10^5 hybridoma recipient cells into a 15-ml conical vial for each infection. Prepare at least one extra tube as an uninfected control. Spin cells, remove supernatant and resuspend cell pellet in 0.5 ml high-titre viral supernatant (> 10^5 cfu ml^{-1}) or in plain medium for the negative control. Place in a single well of a 24-well tissue culture plate. Add 5 µl of 400 µg ml^{-1} polybrene. Incubate for 4–8 h. A longer incubation time increases the infection rate, but decreases cell survival.
3. Transfer the infected cells to a 15-ml conical tube, rinse the well with 1 ml medium and add this to the tube. Spin down the cells and wash once with 1 ml medium.
4. Resuspend cell pellet in 7 ml medium containing 2 mg ml^{-1} G418. Dilute 6×10^3 cells into 20 ml 2× G418 medium (approximately 0.5 ml cells). Plate 100 µl per well on two 96-well plates. This will result in approximately 30 cells per well. Place the remaining cells into a small tissue-culture flask. Repeat steps (3) to (5) for the uninfected control cells. Incubate all cells for 3–4 days.
5. After 4 days of incubation, all uninfected cells should be dead and can be discarded after checking. Flick the medium off the 96-well plates and replace it with 1× G418 medium. After letting the cells in the flask settle, remove approximately 5 ml medium and replace with 5 ml 1× G418.
6. Check the flasks daily. By day 7, the bulk cultures should be mature if the transfection worked well. On day 8 or 9, clones in the 96-well plate should come up and can be screened for transgene expression. For TCR transgenes, measuring IL-2 secretion following stimulation on anti-TCR control plates is a convenient screening method (HT-2 assay (Kappler *et al.*, 1981; Hansen *et al.*, 1989)). To maximize gene expression, grow clones in antibiotic medium keeping the concentration of the cells low (2×10^5 cells ml^{-1} or less).

Although cells can be cloned from the bulk population flask, we have found that they usually do not express the desired gene at high levels. Also, different clones may not represent independent infection events. Instead, the bulk flask is used to monitor the overall success of the transfection before clones may be obvious. TCR transfectants may produce only low levels of transfected genes, but can often be rescued by

fluorescent cell sorting, sometimes requiring several successive rounds of sorting.

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2.3 Killer Cell Assays

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INTRODUCTION

Cell-mediated cytotoxicity plays an important role in the host immune defence against pathogens that reside within cells (Pamer, 1993). Intracellular pathogens escape antibody-, complement- and neutrophil-mediated defences and, therefore, in order to be cleared infected cells have to be specifically identified and destroyed. This role is fulfilled by $CD8^+$ cytolytic T lymphoctes (CTLs), which recognize pathogen-derived epitopes that are presented on the cell surface by major histocompatibility complex (MHC) class I molecules (Zinkernagel and Doherty, 1974). Pathogen-derived proteins are degraded into small peptides (8–10 residues), which are translocated via the transporter associated with antigen processing (TAP) into the endoplasmic reticulum (ER) (Lehner and Cresswell, 1996). Peptides with sufficient affinity for the peptide-binding groove of newly synthesized MHC molecules stabilize MHC–peptide complexes and are transported to the cell surface. $CD8^+$ CTLs detect peptides presented by MHC class I molecules on infected cells with their specific T-cell receptor, resulting in activation of different effector mechanisms that induce death of the infected cell. Two major cytotoxic pathways have been described: (a) release of cytotoxins from secretory granules that directly damage the host-cell membrane (perforins), and (b) induction of programmed cell death (apoptosis) of the target cell by secreted proteases (granzymes) or direct *Fas*/*Fas*–ligand binding (Berke, 1995).

METHODS IN MICROBIOLOGY, VOLUME 25
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We have used the murine model of *Listeria monocytogenes* infection to study the CTL response to infection with an intracellular pathogen. *L. monocytogenes* is a Gram-positive bacterium that survives and multiplies within the cytosol of infected cells. After phagocytosis by macrophages, *L. monocytogenes* lyses the phagolysosomal membrane by secreting listeriolysin (LLO) and enters the host-cell cytosol (Bielecki *et al.*, 1990). Immunocompetent mice infected with a sublethal dose of *L. monocytogenes* clear the infection within a few days and develop long-lasting protective immunity. $CD8^+$ cytotoxic lymphocytes play a major role in this rapid, extremely effective immune response (Kaufmann *et al.*, 1985).

In this chapter we describe different strategies used to characterize CTLs specific for *L. monocytogenes*, to identify pathogen-derived epitopes, and to study the efficiency of MHC class I antigen processing. Although *L. monocytogenes* is relatively easy to work with, we believe that the methods described in this chapter can be readily applied to the study of CTL responses to other intracellular pathogens. Thus, by taking into account the specific characteristics of different intracellular pathogens (e.g. their cell specificity, subcellular localization, persistence and intracellular growth rate), these methods can be modified for detailed studies of CTL responses to other intracellular infections.

◆◆◆◆◆ CHROMIUM RELEASE ASSAY

Several assays have been established to detect and quantify CTL-mediated cell lysis (see later). Because of its high sensitivity and specificity, the standard chromium release assay (CRA) remains one of the best and most convenient methods (Brunner *et al.*, 1968). The principle behind the CRA is to label target cells with radioactive ^{51}Cr. Most cell types take up Na_2CrO_4 when exposed to high concentrations and, as long as they are viable, release the chromium very slowly. Chromium-51 labelled cells can be infected with a pathogen or coated with antigenic peptides and incubated together with CTLs. If the T cells, referred to as effector cells in these assays, detect their specific epitope on the surface of labelled target cells, they induce cell death and damage of the cell membrane, releasing ^{51}Cr into the culture medium. The ratio of released to cell associated ^{51}Cr is proportional to the degree of cell lysis.

Chromium Release Assay using Infected Target Cells

As mentioned above, ^{51}Cr labelled target cells can be infected with intracellular pathogens and tested for lysis by specific CTLs (Kaufmann *et al.*, 1986). These assays can determine if pathogen-specific CTLs are elicited by infection and, if epitope-specific T-cell clones or T-cell lines are available, can characterize epitope presentation in actively infected cells. Although different cell types can be used as target cells in CRA, the cell specificity of the pathogen may limit the range of available target cells. An additional

restriction on the cell type that can be used in the CRA is that the majority of cells must be infected in order for the specific lysis to be interpretable. In the case of *L. monocytogenes*, essentially 100% of bone marrow macrophages and macrophage tumour cell lines can be reproducibly infected. We have studied *Listeria*-specific CTL responses in laboratory mice on the $H2^d$ background (e.g. Balb/c) and have used the $H2^d$ macrophage tumour cell line J774 (ATCC TIB 67) as a target cell (Pamer *et al.*, 1991). J774 cells are readily infected with *L. monocytogenes*, and bacteria enter the cytosol and multiply intracellulary. The cell line is grown in conventional culture medium and does not require supplementary growth factors. Thus, nearly unlimited amounts of target cells are available, and the homogeneity of the tumour cell line makes this system highly reproducible.

A typical protocol for testing *Listeria*-specific CTL lines for specific lysis of J774 cells infected with live bacteria is described below.

Reagents and equipment

- Antibiotic-free culture medium ($RP10^-$): RPMI 1640 supplemented with L-glutamine plus 10% fetal calf serum (FCS)
- [^{51}Cr]sodium chromate (Dupont, MA, USA)
- gentamicin sulfate (Gemini Bio-Products, CA, USA)
- 0.5% Triton X-100
- 96-well U-bottomed microtitre plates
- J774 macrophage cell line (ATCC TIB 67), cultured in $RP10^-$
- bacterial culture: virulent *L. monocytogenes* (e.g. ATCC 43251) grown in trypticase soy broth (TSB)
- γ counter
- effector cells: *Listeria*-specific T-cell line.

Making chromium-51 labelled target cells

J774 cells are labelled by short incubation in the presence of high concentrations of ^{51}Cr. Chromium-51 has a relatively short half-life (28 days) and is less hazardous to work with than most other isotopes. Nevertheless, radiation safety training is necessary for every person working with ^{51}Cr. All objects must have radioactive material warning labels, and radioactive waste must be collected and disposed of in specially designated containers. A Geiger counter should be used to check for contamination.

1. Pellet 1×10^6 J774 cells (500*g*, 5 min).
2. Resuspend cells in 100 µl $RP10^-$.
3. Add 100 µCi [^{51}Cr]sodium chromate (usually equivalent to 100 µl, but calculate the actual activity considering a half-life of 28 days).
4. Incubate for 1 h at 37°C.
5. Wash cells twice in 10 ml $RP10^-$.
6. Resuspend cells in 10 ml $RP10^-$ ($= 1 \times 10^5$ cells ml^{-1}).
7. Add 100 µl ($= 1 \times 10^4$ cells) per well in a 96-well plate and allow macrophages to adhere for 30 min at 37°C.

Infecting labelled cells with intracellular bacteria

Mid-log-phase *L. monocytogenes* are added directly to ^{51}Cr-labelled macrophages. After incubation, the medium is replaced by RP10$^-$ containing gentamicin, a membrane-impermeable antibiotic, to kill extracellular, but not intracellular bacteria.

1. Grow *L. monocytogenes* in TSB to early/mid-log-phase, to an A_{600} of 0.1. At this density there are approximately 2×10^8 bacteria per millilitre.
2. Add 10 µl bacteria ($=2\times10^6$) to wells with ^{51}Cr labelled J774 cells designated as infected target cells.
3. For uninfected controls add 10 µl TSB.
4. Incubate for 25 min at 37°C.
5. Carefully remove 80 µl of medium from each well and replace with 80 µl RP10$^-$ containing 10 µg ml^{-1} gentamicin.

Assaying for specific lysis with CD8$^+$ T cells using different effector/target ratios

To assay for specific lysis, CD8$^+$ T cells are incubated together with the prepared target cells (generation of antigen-specific CTLs is described in the chapter by Born *et al.*). The more antigen-specific T cells/effector cells are added to the assay, the higher the expected extent of specific target cell lysis. However, higher effector/target (E/T) ratios are often accompanied by an increase in non-specific target cell lysis. Antigen-specific lysis is determined by comparison of the degrees of lysis in parallel incubations of effector cells with infected and uninfected target cells. Since the optimum E/T ratio is difficult to predict, it is advisable to test for specific lysis at several E/T ratios. Usually, the number of target cells is kept constant (here 1×10^4 cells) and an E/T ratio titration is achieved by adding different dilutions of effector cells. A titration of lytic activity by increasing the number of effector cells that is clearly higher (>10–20%) than the uninfected control indicates antigen-specific lysis. A typical CTL assay contains the following controls and titrations.

Spontaneous release

Infected and uninfected labelled target cells are incubated in the absence of effector cells to control for the spontaneous release of chromium.

1. Add 100 µl RP10$^-$ to four wells of uninfected and infected target cells.

Maximum release

Labelled target cells are lysed by adding a detergent to estimate the maximum radioactivity that can be released.

1. Add 100 µl 0.5% Triton X-100 to four wells.

CTLs plus infected or uninfected target cells

CTLs are tested for lysis of uninfected and infected ^{51}Cr labelled target cells at various E/T ratios to detect specific and background lysis.

1. Add 100 µl effector cells (in $RP10^-$) to infected and uninfected target cells. Make several dilutions of effector cells to achieve E/T ratios of 30 : 1, 10 : 1, 3 : 1, 1 : 1 and 0.3 : 1.
2. The final volume in all wells should be 200 µl.
3. Incubate the cells for 3 h at 37°C in a 5% CO_2 incubator.
4. Pellet the cells in the 96-well plate by gentle centrifugation at 400*g* for 5 min.
5. Carefully harvest 100 µl of supernatant from each well with a multichannel pipettor and count the released ^{51}Cr with a γ counter.

Determining the percentage specific lysis

Specific lysis is calculated by accounting for spontaneous chromium release SR and maximum release MR using the formula:

$$\% \text{ Specific lysis} = \frac{100 \times \text{cpm sample} - \text{cpm SR}}{\text{cpm MR} - \text{cpm SR}}$$

where cpm is counts per minute. Lysis that is specifically related to the presence of the pathogen can be estimated by comparing the values of percentage specific lysis for infected versus uninfected target cells at the same effector/target ratios (Fig. 1).

Limitations of direct CTL assays

Using target cells infected with intracellular pathogens as CTL targets has certain disadvantages that often limit the utility of these assays. For example:

- The pathogen continues to multiply inside the infected target cell. Pathogens with a high intracellular growth rate might simply burst the infected cell within a relatively short time period, resulting in a rapid increase in spontaneous ^{51}Cr release. Alternatively, some intracellular pathogens release lytic proteins that can cause high degrees of spontaneous lysis in the absence of CTL. If the spontaneous release of ^{51}Cr exceeds 30–40% of maximum release values, then the specific lysis values become very difficult to interpret.
- Although the incubation time for CTL assays could be shortened to prevent exceedingly high spontaneous release values, there is a certain delay until specific target cell lysis is detectable. Thus, this strategy is limited to curtailing the length of incubation to a minimum of 2–3 h. Possible explanations for these delayed kinetics are that specific CTLs need time to find their target cells and to induce cell death, and that target cells may not immediately release all ^{51}Cr upon encounter with a specific CTL.

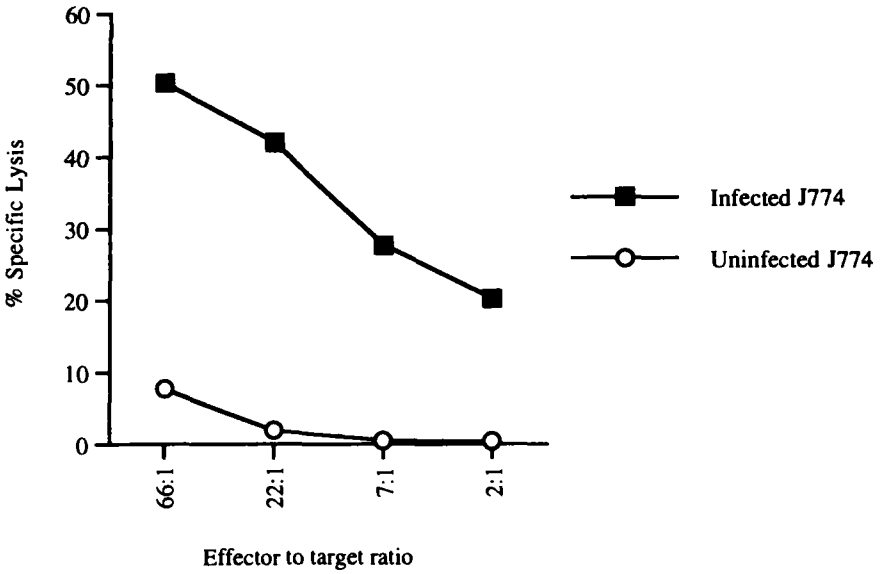

Figure 1. CTL derived from *L. monocytogenes* immunized mice specifically lyse infected J774 cells. Spleens cells from *L. monocytogenes* immunized Balb/c mice were restimulated *in vitro* with infected J774 cells. Five days following *in vitro* restimulation, CTL were assayed for specificity using *L. monocytogenes* infected or uninfected ^{51}Cr labelled J774 cells, as described in the text. The percentage specific lysis was determined 3 h after addition of different CTL numbers to 10000 target cells.

Chromium Release Assay using Target Cells coated with Peptide Extracts of Infected Cells

Several years ago Rammensee and colleagues (Roetzschke *et al.*, 1990) exploited the ability to acid elute and purify by high performance liquid chromatography (HPLC) MHC associated peptides from infected cells to transfer them to uninfected cells and assay for antigen-specific CTL mediated lysis. Although peptide–MHC class I complexes are relatively stable at physiological pH, at low pH peptides rapidly dissociate. Pathogen-derived epitopes can be eluted directly from infected cells by exposure to a pH of 2.0 using 0.1% trifluoroacetic acid (TFA). Eluted peptides are separated from high molecular weight material and fractionated by reverse-phase HPLC and ^{51}Cr labelled target cells are coated with HPLC purified peptide fractions and tested for specific lysis in a standard CTL assay. This procedure has several advantages over direct CTL assays with infected target cells (see above):

- Epitopes can be extracted from large numbers of infected cells and then added in a relatively high concentration to target cells, increasing the sensitivity of the assay.
- Target cells can be chosen (i.e. cells with high ^{51}Cr uptake, low spontaneous release and a high capacity to bind exogenously added peptides) that optimize sensitivity and result in highly reproducible results.

- In the case of pathogens that infect cells that cannot be used in CTL assays, peptides can be extracted and transferred to ^{51}Cr labelled cells.
- Pathogens that lyse cells within the time frame of a conventional CTL assay can be studied by extracting epitopes from cells that are damaged, since epitopes are protected from proteolysis by the MHC molecule even in dead cells.
- HPLC fractionation of antigenic epitopes allows one to estimate the complexity of the CTL response, assuming that most CTL epitopes will elute in different fractions.
- By loading partially MHC-mismatched target cells, the MHC restriction for individual epitopes can be determined.
- Purified peptides can be sequenced to obtain more detailed information about the structure and origin of epitopes.

Reagents and equipment (see above)

- Tissue culture plates (e.g. Falcon 3025, 150 mm).
- 0.1% trifluoracetic acid.
- Dounce homogenizer and sonicator.
- Centricon 10 (Amicon, MA, USA).
- Ultracentrifuge.
- HPLC with C18-300A column (Delta Pak, 3.9×300 mm, 15 mm spherical beads).
- Lyophilization unit.
- A cell line that can be infected *in vitro* with the intracellular pathogen (e.g. macrophage cell lines J774, IC21, PU51R, RAW264.7, etc.).
- Target cells: usually a tumour cell line expressing the appropriate MHC class I molecules (e.g. $H2^d$ positive mastocytoma cell line P815/ATCC TIB64).

Harvesting large numbers of cells infected with pathogenic organisms

In the *L. monocytogenes* system we use the macrophage cell line J774, which can be easily cultured and expanded *in vitro*. Large numbers of cells are grown in tissue culture plates and infected with the pathogen. After cellular infection, cells are incubated for various time intervals before harvesting for peptide extraction. Analysis at different time points is not only necessary to search for optimal epitope extraction times, but also reveals important information about the kinetics of antigen presentation (see later).

1. J774 cells are grown to confluence (approximately 10^8 cells per plate) in 150 mm tissue cultures plates in 27 ml $RP10^-$ (one plate for each time interval examined).
2. For infection with *L. monocytogenes* add 3 ml of mid-log-phase culture in TSB at A_{600} of 0.1 (= 6×10^8 bacteria per plate).
3. Incubate for 30 min at 37°C.

(*cont.*)

(*cont.*)

4. Replace medium with RP10$^-$ containing 5 μg ml^{-1} gentamicin.
5. Incubate for different time intervals (e.g. 1, 3, 5, 7 h).
6. Remove medium and harvest cells by scraping them into 5 ml phosphate buffered saline (PBS) (10 μl cells can be taken, diluted in PBS containing 0.1% Triton X-100, and plated out on TSB agar to estimate the number of bacteria per infected cell at a given time point).
7. Harvested cells are pelleted by centrifugation and pellets are stored at –80°C.

Lysing infected cells and eluting peptides with TFA

Pellets are resuspended in 0.1% TFA and homogenized to extract peptides from the infected cells. Insoluble material is pelleted by high-speed ultracentrifugation, and supernatants are depleted of high molecular weight molecules by passage through a Centricon-10 membrane.

The following steps should be done keeping the samples on ice

1. Resuspend pellets in 10 ml 0.1% TFA.
2. Homogenize cell suspension by dounce homogenization (roughly 20 strokes) and sonication (twenty 1-s cycles at an intermediate setting).
3. Centrifuge for 30 min at 100 000*g*.
4. Freeze supernatant and lyophilize.
5. Resuspend lyophilized material in 2 ml 0.1% TFA and pass the suspension through a Centricon-10 membrane (5000*g*).

HPLC fractionation of infected cell extracts

The material that passes through the Centricon-10 membrane is applied to a reverse-phase HPLC C18 column. The C18 column is eluted with a 0–60% acetonitrile gradient in 0.1% TFA (0–5 min, 0% acetonitrile; and 5–45 min, 0–60% acetonitrile). Fractions (1 ml) are collected at 1-min intervals and lyophilized. Most MHC class I associated peptides elute from the C18 column between 24 and 40 min if this gradient is used. Of course, if the gradient is changed the elution time for peptides will be altered. It is possible to maximize the separation of different peptides by extending the acetonitrile/water gradient.

CTL assay

Peptide fractions are resuspended in PBS and used to coat target cells in a standard chromium release assay. Generally, fractions obtained from 100 million extracted cells are resuspended in 200 μl PBS. Most tumour cell lines express well characterized MHC class I molecules and many of them

show excellent qualities for chromium labelling. Thus, a wide range of possible target cells for different animal models and MHC haplotypes is available. For our system, we take the mouse mastocytoma cell line P815 (ATCC TIB 64) to test for H2-K^d-restricted epitope presentation (Fig. 2).

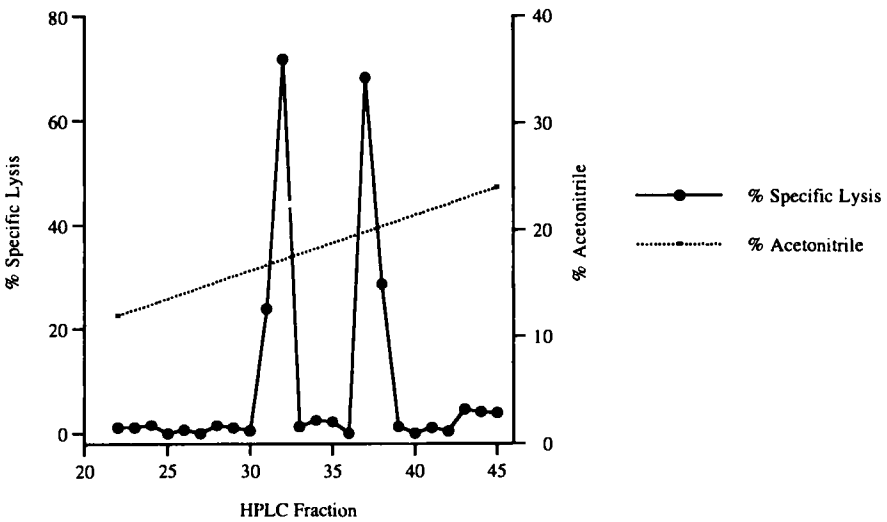

Figure 2. CTL epitopes can be TFA extracted from *L. monocytogenes* infected J774 cells. 400 million J774 cells were infected with *L. monocytogenes* for 6 h and then harvested and TFA extracted as described in the text. Low molecular weight material was applied to a C18 reverse-phase HPLC column and eluted with a shallow gradient of acetonitrile. HPLC fractions were lyophilized, resuspended in PBS and assayed for recognition by an *L. monocytogenes* specific, non-clonal T-cell line. P815 ($H2^d$) cells were labelled with ^{51}Cr and used as targets. The effector/target ratio in this experiment was 10:1. It can be seen that this CTL line recognizes two different peptides that elute from the C18 column with different acetonitrile concentrations.

1. For detailed information, see earlier.
2. P815 cells are labelled with ^{51}Cr, and 1×10^4 cells in 50 µl RP10 are placed in wells of a 96-well plate.
3. HPLC fractions are resuspended in 200 µl of PBS; 50 µl of each sample is added to a designated well of target cells.
4. Incubate for 45–60 min at 30°C.
5. Add CTLs at a constant E/T ratio in a volume of 100 µl RP10 medium to the well (to determine an optimal E/T ratio, different effector cell dilutions should be tested in advance for specific and unspecific lysis).
6. Incubate plates for 4–6 h at 37°C and harvest supernatants as described.

Extraction of MHC class I associated peptides from infected spleens

Essentially all studies of antigen processing have been performed *in vitro*, in systems that are, at best, approximations of *in vivo* events. Studies of *in vivo* antigen processing are difficult, however, since the quantity and concentration of antigen is very low. It is possible, however, to isolate MHC class I associated peptides from infected spleens and to identify among these peptides pathogen-derived epitopes (Pamer *et al.*, 1991). In the *L. monocytogenes* system, these experiments are generally only successful when mice are infected with a very high infectious dose and when peptides are extracted from spleens 48 h after infection. Because of the great potential of this method for directly correlating *in vivo* antigen processing with T-cell responses, we briefly describe how to acid extract *Listeria*-derived peptides from infected spleens.

1. Balb/c mice are infected intravenously with 1×10^6 virulent *L. monocytogenes* (ATCC 43251).
2. At 48 h after infection, one or two spleens are taken and homogenized in 10 ml 0.1% TFA with a ground glass tissue grinder followed by dounce homogenization and sonication, as described above for infected J774 cells. The pH should be checked with pH paper, and if greater than 2.0 should be adjusted with 1% TFA to pH 2.0.
3. The homogenate is centrifuged for 30 min at 100 000*g*.
4. The supernatant is lyophilized and then resuspended in 2 ml 0.1% TFA.
5. The resuspended extract is passed through a Centricon-10 membrane. Because this extract can be quite viscous, passage through the membrane may require 3–4 h of centrifugation.
6. The Centricon-10 filtrate is HPLC fractionated and individual fractions are lyophilized and resuspended in PBS and used in CTL assays as described earlier.

◆◆◆◆◆◆ QUANTITATION OF MHC CLASS I ASSOCIATED EPITOPES IN INFECTED CELLS

Falk *et al.* (1991) were the first to determine the number of virus-derived epitopes in influenza virus infected cells by TFA extracting and quantifying epitopes. This powerful method for quantifying the end result of the MHC class I antigen-processing pathway has been extended to *L. monocytogenes* infected cells (Villanueva *et al.*, 1994) and to cells expressing human immunodeficiency virus (HIV) proteins (Tsomides *et al.*, 1994). To quantify natural epitopes in infected cells successfully, it is necessary to:

- Obtain purified and precisely quantified synthetic peptides.
- Determine the efficiency of peptide extraction from cell pellets.
- Generate a standard curve that determines the percentage specific lysis with a particular CTL clone relative to the peptide concentration in the CTL assay.
- Isolate natural CTL epitopes from a uniformly infected population of cells by TFA extraction and HPLC purification and determine the specific lysis obtained with epitope containing HPLC fractions.
- Determine the concentration of epitope in the HPLC fraction by comparing the percentage specific lysis to the synthetic peptide standard curve.
- Using the starting number of infected cells that were extracted and correcting for the extraction efficiency for the CTL epitope, calculate the number of epitopes that were extracted per individual cell.

In the following sections we provide detailed information on each of these steps.

Precise Quantitation of Synthetic Peptides

It is essential to use highly purified synthetic peptides for epitope quantitation. Since synthetic peptides frequently contain truncated versions of the peptide, HPLC purification is necessary following synthesis. This can be accomplished by subjecting approximately 100–200 μg of synthetic peptide to reverse-phase HPLC fractionation, using a gradient of water and acetonitrile with 0.1% TFA, as described in the previous section. The synthetic epitope should be readily identified as the predominant peak absorbance at 212 nm, collected and lyophilized. It is important to remember that CTL can detect very small quantities of synthetic peptide. Thus, when purifying large quantities of synthetic peptide by HPLC, it is likely that the HPLC apparatus will become contaminated with the peptide. Subsequent runs on the same HPLC system will contain trace quantities of epitope that will interfere with the analysis of TFA extracts from infected cells. For this reason we advise always purifying synthetic epitopes on a separate HPLC system than the one used for fractionating TFA extracts from infected cells.

The HPLC purified epitope should be assayed by mass spectrometry to determine the purity and mass of the peptide. The most accurate way to quantify the peptide is to subject it to hydrolysis and perform quantitative amino acid analysis. Although other methods of quantifying peptides are available, they are highly dependent on the amino acid content of the peptide, and thus are difficult to standardize.

Determining the Extraction Efficiency of Epitopes from Cellular Pellets

Different peptides, depending on their hydrophobicity, stability and sensitivity to proteases are likely to be extracted from infected cell pellets with different efficiencies. Furthermore, since different peptides elute in different HPLC fractions, the antigenic peptides will be competing with

different endogenous peptides for MHC class I binding in subsequent CTL assays. Therefore, it is important to determine the overall efficiency of peptide detection following TFA extraction from cells. This is accomplished by spiking a pellet of 100–200 million cells with a small quantity of synthetic epitope (20, 50 and 100 µl of 10^{-10}M synthetic peptide) and TFA extracting the spiked pellet, as described in the previous section. Following HPLC fractionation, the amount of peptide that is present in the epitope containing fractions is titrated and compared to the synthetic peptide that was used to spike the pellet, and the yield can be estimated. We have found that the yield for different peptides generally falls in the range 20–80%. It is important to redetermine the efficiency of peptide extraction and detection for individual epitopes if the HPLC gradient is changed, since changes in the gradient will change the family of endogenous peptide that the antigenic peptide travels with. In general, we find that the extraction efficiency for epitopes improves as the acetonitrile gradient is made shallower.

Preparing Synthetic Epitope Standard Curves

MHC class I restricted CTL clones detect target cells in the presence of very low concentrations of synthetic peptide epitopes. However, the sensitivity of CTL clones for their antigenic peptides varies. Some clones are more sensitive than others. Depending on the length of time since CTL clones were last stimulated, their sensitivity to peptide and the percentage specific lysis they induce can also vary. Thus, when

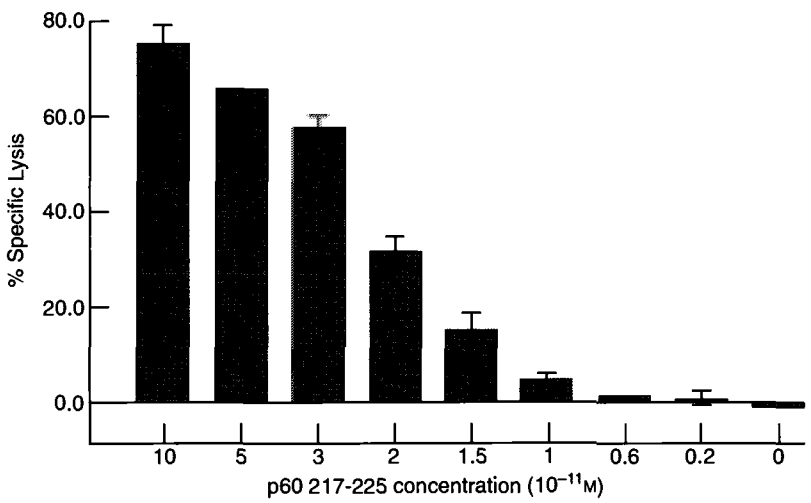

Figure 3. Percentage specific lysis correlates with the peptide concentration present in the CTL assay. P815 target cells were labelled with ^{51}Cr and incubated in different concentrations of precisely quantified p60 217–225, an H2-K^d restricted CTL epitope. Target cells were assayed for recognition by CTL clone L9.6, which is specific for p60 217–225, at an effector/target ratio of 3 : 1. After 4 h the percentage specific lysis was determined and plotted against the peptide concentration.

quantifying the amount of epitope in TFA extracts from infected cells, it is critical to determine concurrently the sensitivity of the CTL using known concentrations of synthetic peptide. Our *L. monocytogenes* specific CTL clones detect their epitopes at concentrations as low as 10^{-12} M, and generally maximally lyse target cells at concentrations greater than 5×10^{-11} to 10×10^{-11} M epitope. Therefore, we have made dilutions of the synthetic peptide in the range 10^{-10} to 10^{-13} M, which is a concentration range where the percentage specific lysis correlates with the epitope concentration used to coat the target cells. In a typical assay, we generate a standard curve using epitope concentrations of 100, 80, 60, 40, 20, 10, 8, 6, 4, 2 and 1×10^{-12} M, and assay in triplicate each of these concentrations with a specific CTL clone (Fig. 3).

Calculating the Quantity of CTL Epitopes in TFA Extracts of Infected Cells

The first step in quantifying epitopes in infected cells is to determine the epitope concentration in HPLC fractions of TFA extracts of infected cells. This is done by assaying, in triplicate, an amount of the HPLC fraction (generally 2–50 µl) that gives detectable but less than maximal lysis in a CTL assay. It is important that the percentage specific lysis obtained with HPLC fractions falls within the range of the standard curve. By comparing the percentage specific lysis obtained with HPLC fractions to that obtained with the standard curve it is possible to determine the epitope concentration in the HPLC fraction. By factoring in the volume that was assayed, it is possible to determine the molar amount of epitope that is present in a given HPLC fraction.

Estimating the Number of CTL Epitopes per Cell

The average number of CTL epitopes present per cell can be determined by taking into account the starting number of infected cells, the extraction efficiency of the epitope from infected cells, the concentration of the epitope in the HPLC fraction and the volume of the HPLC fraction. Using Avogadro's number, it is possible to convert the molar amount of epitopes A into the absolute number. The following formula takes these factors into account:

$$\text{Epitopes per cell} = \frac{A \times Y \times (6.02 \times 10^{23})}{\text{No. of J774 cells}}$$

where Y is a factor to correct for the extraction efficiency of the epitope.

Thus, if the extraction efficiency is 20%, $Y=5$. Alternatively, if the extraction efficiency is 50%, then $Y=2$. Using this formula, it is possible to determine the numbers of different epitopes that are present per cell, at different times of infection or under varying circumstances (see below).

◆◆◆◆◆◆ KINETIC ANALYSES OF MHC CLASS I ANTIGEN PROCESSING IN INFECTED CELLS

The kinetics of antigen processing and presentation can be studied by quantifying epitope numbers at varying time intervals during the course of cellular infection. CTL epitope generation and presentation is a dynamic process that requires degradation of pathogen-derived proteins, transport of peptides into the endoplasmic reticulum (ER), loading of empty MHC class I molecules and translocation to the cell surface. Each step is characterized by distinct specificities, kinetics and efficiencies that may differ from epitope to epitope. Furthermore, the overall number of epitopes present in an infected cell at a given time point also reflects peptide losses that may result from, for example, degradation by cytosolic or ER proteases prior to binding by MHC class I molecules or from rapid dissociation from MHC class I molecules following binding. To study the kinetics of class I antigen presentation in more detail, the pathway can be blocked at distinct points and the effect on epitope generation determined. An advantage of studying antigen processing in cells infected with bacteria is that the prokaryotic and eukaryotic protein syntheses can be inhibited independently. Thus, bacterial antigen synthesis can be turned off at defined time points and the fate of the remaining antigen investigated. Furthermore, specific inhibitors of host cell proteolysis, protein synthesis and membrane trafficking can be used to examine their impact on the MHC class I antigen processing pathway, without affecting the production of antigen by the intracellular bacterium.

Isolation and Quantitation of CTL Epitopes from Cells Infected for Varying Time Intervals

The number of epitopes per cell can be calculated by acid elution and HPLC fractionation as described earlier. Following this approach quantitative analyses can be performed at different time points during infection with the pathogen. We have used this approach to study antigen processing in J774 cells infected with *L. monocytogenes* for 1, 2, 3, 4, and 5 h (Villanueva *et al.*, 1994). Epitope numbers and numbers of bacteria per cell were determined as described (see earlier), and it was demonstrated that there is a direct correlation between the secretion of antigenic proteins into the host-cell cytosol and the generation of CTL epitopes.

Use of Inhibitors of Bacterial Protein Synthesis to Examine MHC Class I Antigen Processing

As mentioned above, prokaryotic protein synthesis can be specifically inhibited without affecting host-cell metabolism. The antibiotic tetracycline (TCN) is such an inhibitor with a wide spectrum for Gram-positive and Gram-negative bacteria. Unlike gentamicin, which is membrane impermeable and can be used to kill extracellular bacteria, TCN is mem-

brane permeable and, therefore, inhibits protein synthesis of intracellular bacteria. TCN rapidly inactivates bacterial ribosomes, terminating intracellular bacterial protein synthesis within several minutes of addition to the culture medium. To investigate the linkage between antigen synthesis and epitope generation in macrophages infected with bacteria, J774 cells were grown to confluence in 150-mm culture plates and infected with *L. monocytogenes*, as described in the preceeding sections. After 3 h of infection, TCN (20 μg ml^{-1}) was added, and control plates were left untreated. At different time intervals (e.g. 4, 5, 6 and 7 h post-infection) TCN-treated and untreated cells were scraped into PBS and epitopes were extracted and determined as described (see earlier). Using this system, we found that in infected cells treated with TCN the generation of epitopes is markedly and rapidly diminished, indicating that epitopes derive from newly synthesized antigens (Villanueva *et al.*, 1994).

Using Inhibitors of Host Cell Proteolysis, Protein Synthesis and Protein Transport to Study Parasite Interactions with the MHC Class I Antigen Processing Pathway

Antigen processing and epitope presentation by the host cell is a multistep pathway that requires antigen degradation by cytosolic proteases, transport of peptide fragments into the ER, synthesis of MHC class I molecules, and transport of epitope–MHC class I complexes to the cell surface. Specific inhibitors that interfere with distinct steps in this pathway are available and provide an opportunity to study the contribution of these steps to the efficiency of epitope generation. In the following sections we briefly summarize some of these approaches.

Inhibitors of host cell proteolysis

Cytosolic degradation of pathogen-derived antigens is mainly mediated by proteasomes, and distinct peptidase activities have been associated with these multicatalytic enzyme complexes (Orlowski *et al.*, 1993). Peptide aldehyde protease inhibitors, e.g. *N*-acetyl-Leu-Leu-norleucinal (LLnL) and (benzyloxycarbonyl)Leu-Leu-phenylalaninal (Z-LLF) have been reported to inhibit specifically chymotrypsin-like activity of proteasomes (Rock *et al.*, 1994). These inhibitors are membrane permeable and can be used to treat pathogen-infected cells. We have found, in cells infected with *L. monocytogenes*, that these inhibitors do not impair host cell or bacterial protein synthesis. To determine the impact of proteasome inhibition on the generation of CTL epitopes in *L. monocytogenes* infected J774, we infected cells as described in the preceding section and treated infected cells with either 250 μM LLnL or 10 μM Z-LLF. Infected cells were harvested at varying time points and epitopes were extracted, purified by HPLC and quantified, as described in the previous sections. Using this approach it was possible to determine that protein degradation of secreted bacterial proteins is tightly linked to epitope generation (Sijts *et al.*, 1996). Furthermore, the fact that the generation of different epitopes

varied in sensitivity to these protease inhibitors suggests that more than one degradation pathway is used to generate different MHC class I associated epitopes.

Inhibitors of host cell protein synthesis

As mentioned previously, MHC class I antigen processing involves multiple steps that occur in separate subcellular compartments. In order to determine the requirements of host-cell protein synthesis for efficient CTL epitope generation, it is possible to inhibit selectively host-cell protein synthesis with 50 μg ml^{-1} cycloheximide (CHX) and 30 μg ml^{-1} anisomycin (ANM) without affecting intracellular bacterial protein synthesis (Sijts and Pamer, 1997). As expected, inhibiting host-cell protein synthesis in the *L. monocytogenes* system rapidly inhibits CTL epitope generation because of the rapid depletion of available MHC class I molecules. Surprisingly, however, inhibiting host-cell protein synthesis does not impair either the degradation of bacterial proteins in the host-cell cytosol, transport of peptides into the ER or trafficking of MHC class I molecules to the cell surface.

Inhibitors of intracellular trafficking

After loading of MHC class I molecules in the ER, MHC–peptide complexes are transported rapidly via the Golgi complex to the cell surface. BrefeldinA (BFA) is an antibiotic that disrupts the Golgi complex, thereby preventing the translocation of ER contents to the cell surface. In the *L. monocytogenes* system, treatment of infected cells with 5 μg ml^{-1} of BFA does not affect intracellular bacterial growth, protein synthesis or protein secretion (Sijts and Pamer, 1997). Thus, it is possible to study and compare the interaction of CTL epitopes with MHC class I molecules on the cell surface or in the ER.

◆◆◆◆◆◆ OTHER ASSAYS THAT ARE USEFUL FOR INVESTIGATING INTRACELLULAR PATHOGEN SPECIFIC CTL

In some systems it can be very difficult to infect target cells and concurrently label them with ^{51}Cr. Therefore, other methods have been established to quantify CTL-mediated lysis of infected target cells. In the following sections we briefly outline some of these methods, and refer the reader to appropriate references for detailed protocols.

Serine Esterase Release

Activated CTLs release cytotoxins and proteases from secretory granules, which induce death of the target cells. One of the proteases is the enzyme

serine esterase, which can be detected in the culture medium upon its release from CTLs (Taffs and Sikovsky, 1994). Enzyme activity correlates with the extent of CTL activation in the presence of epitope-presenting target cells. The detection of serine esterases is based on hydrolysis of *N*-α-benzyloxycarbonyl-L-lysine thiobenzyl ester, which is detected in a standard colorimetric assay using dithio-bis(2-nitrobenzoic acid). In this assay the amount of enzymatically active serine esterase that is released by antigen stimulation is compared to release in the absence of antigen and the maximal release obtained with a mild detergent. Thus, this assay is similar to conventional ^{51}Cr release assays, except that the condition of the target cell prior to interaction with CTL is less critical.

Sensitivity to Membrane-impermeable Antibiotics

When CTLs attack a target cell, perforins damage the target cell membrane and create pores that allow otherwise impermeable substances to enter the cell (Berke, 1995). In the *L. monocytogenes* system it is possible to take advantage of this to determine antigen-specific CTL activity. Specifically, target cells are infected with *L. monocytogenes* and the CTL assay is performed in medium containing the membrane-impermeable antibiotic gentamicin (Bouwer *et al.*, 1992). In the absence of CTLs, or if the CTLs do not detect *L. monocytogenes* antigens, intracellular bacteria survive inside target cells and can be quantified by plating on TSB culture plates. If CTL lysis of infected cells occurs, however, the number of bacteria that can be cultured is diminished, since gentamicin enters the lysed, infected cells and kills intracellular bacteria. Although this method is less precise than most other CTL assays, it provides an alternative to ^{51}Cr release assays and the problem of bacteria-induced ^{51}Cr release. One potential problem with this method is that infected cells can begin to 'leak' after 4–5 h of infection, allowing the influx of antibiotics into target cells (see earlier).

[^{3}H]Thymidine-based CTL Assay

Cytolytic T cells induce apoptosis in target cells, and the fragmentation of target cell DNA can be used to measure antigen-specific recognition (Duke and Cohen, 1994). To perform this assay, the DNA of target cells is labelled with [^{3}H]thymidine prior to interaction with CTLs. DNA fragmentation of the target cells is measured as release of ^3H into the culture medium following addition of CTLs. Specific lysis can be determined by measuring soluble ^3H in the supernatant and cell-associated ^3H in the cell pellet in the absence and presence of CTLs. The strength of this assay is a relatively low spontaneous release, allowing incubations for 10 h and more. Thus, direct lysis of cells by perforin mediated mechanisms can be detected as well as lysis by *Fas/Fas*–ligand interactions, which generally follows a longer time course. Utility of this assay will be limited if the pathogenic organism induces apoptosis in the target cell.

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2.4 Measuring Cytokine Responses by ELISA, ELISPOT and RT-PCR Methods

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◆◆◆◆◆◆ INTRODUCTION

In general, $CD4^+$ T-helper (Th) cells are subdivided into at least two subsets, namely Th1 and Th2 cells, according to distinct cytokine profiles which accounted for two major functions, cell mediated immunity (CMI) and humoral immunity in host immune responses, respectively (Mosmann and Coffman, 1989; Street and Mosmann, 1991). It is well established that Th1 cells secrete interleukin-2 (IL-2), γ-interferon (IFN-γ) and tumour necrosis factor β (TNFβ) and function in CMI for protection against intracellular bacteria and viruses. Th1 cells also provide limited help for B cell responses, where IFN-γ supports immunoglobulin G2a (IgG2a) synthesis in mice. The Th2 cells preferentially secrete IL-4, IL-5, IL-6, IL-10 and IL-13, and provide effective help for B cell responses, in particular for IgG1 (and IgG2b), IgE and IgA synthesis (Bond *et al.*, 1987; Coffman *et al.*, 1987; Murray *et al.*, 1987; Beagley *et al.*, 1988, 1989; Harriman *et al.*, 1988; Lebman and Coffman, 1988; Fujihashi *et al.*, 1991). For the generation of these two subsets of regulatory Th cells, several different cytokines can influence the process of development of Th1 and Th2 cells. For example, IL-12 and IL-4 may direct $CD4^+$ Th cell development

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down a Th1 or Th2 pathway, respectively, while later in development IFN-γ and IL-10 (together with IL-4) can reinforce Th1 or Th2 phenotype expansion (Seder and Paul, 1994).

It is now essential to measure the level of Th1 and Th2 cytokines in order to elucidate the exact mechanism for the induction and regulation of antigen-specific immune responses. The current technology allows the detection of Th1 and Th2 cytokines at the level of the protein, the cell or the mRNA. For the measurement of secreted cytokines, two distinct methods are currently available. Biological activity of cytokines can be measured by using certain cytokine-dependent cell lines (Slavin and Syrober, 1978; Watson, 1979; Helle *et al.*, 1988; Sawamura *et al.*, 1990). Although this assay is the most sensitive way to detect biologically active cytokines, the enzyme-linked immunosorbent assay (ELISA) system is a simple, rapid and sensitive assay for the quantitative analysis of different cytokines using appropriate combinations of cytokine-specific monoclonal antibodies. Thus, cytokine-specific ELISA is now widely used for the quantitation of cytokines in both *in vivo* and *in vitro* investigations. In addition to cytokine-specific ELISA, enzyme-linked Immunospot (ELISPOT) assay is also available for the elucidation of Th1 and Th2 cytokine producing cells. An advantage of cytokine-specific ELISPOT assay is that it is able to determine the frequency of cytokine-producing cells in the single-cell preparation (Taguchi *et al.*, 1990a,b; Fujihashi *et al.*, 1993a). Thus, the frequency and number of Th1 and Th2 cytokine-producing cells in different cell fractions (e.g. $CD4^+$ and $CD8^+$) from various tissues (e.g. mucosal and systemic) can be determined.

In addition to cytokine-specific ELISA and ELISPOT assays, which detect cytokine at the protein and cellular levels, respectively, Th1 and Th2 cytokines can be analysed at the molecular level by detecting cytokine-specific mRNA using northern blot, *in situ* hybridization, dot blot, mRNA protection and reverse transcriptase polymerase chain reaction (RT-PCR) assays. Of these assays, RT-PCR is the most rapid and efficient method for the detection of Th1 and Th2 specific mRNA, especially when dealing with small numbers of specific subsets of lymphocytes. Furthermore, the recent development and adoption of quantitative RT-PCR has enhanced the capability to characterize increases or decreases in specific cytokine message (Wang *et al.*, 1989; Hiroi *et al.*, 1995; Marinaro *et al.*, 1995). This chapter introduces the most up-to-date cytokine-specific ELISA, ELISPOT and RT-PCR methods for murine and primate models, which are routinely performed in our laboratories in order to detect cytokine synthesis at the protein, single-cell or mRNA level. As a human-specific assay is described on pages 621–650, this chapter focuses on the cytokine-specific ELISA, ELISPOT and RT-PCR assays for non-human primate systems, since these experimental animal models are important and useful in characterizing acquired immune deficiency syndrome (AIDS) specific immunity.

◆◆◆◆◆◆ MEASURING MURINE CYTOKINES

Background

Cytokines are important immunoregulatory proteins that mediate distinct functions of different immunocompetent cells including T and B cells, macrophages, dendritic cells and natural killer (NK) cells. Th1 and Th2 cytokines were produced by stimulation of T cells via specific interactions with antigen-presenting cells (APCs) through a two-step signalling pathway via the T-cell receptor (TCR) and major histocompatibility complex (MHC) class II with processed peptide, as well as through CD28 and B7-1/B7-2 molecule interactions. Techniques designed to detect cytokine expression have proven valuable in studies of immune responses against different infectious diseases and for developing effective vaccines. We discuss three of these techniques for detecting murine cytokines: cytokine-specific ELISA and ELISPOT, and quantitative RT-PCR. Cytokine-specific ELISA and ELISPOT assays are used to measure cytokines at the protein and cellular levels, respectively. The former assay measures secreted cytokines, while the latter system is used to quantitate the number of cells producing a specific cytokine. Finally, RT-PCR is used to measure the expression of cytokine at the mRNA level.

Mouse-specific ELISA

ELISA protocol

The murine cytokine-specific ELISA assays are highly specific, simple and rapid procedures for the quantitative analysis of secreted cytokines. This assay quantitates the cytokines produced by Th1 and Th2 type cells in culture supernatants and body fluids, including serum and external secretions. Although ELISA is a powerful technique for assessing the exact levels of accumulated cytokines in culture supernatants, one must carefully interpret the results obtained using this method. For example, it is possible that some cytokines produced by $CD4^+$ Th cells may be consumed by neighbouring cells during the culture period. However, this is the most commonly used method for the characterization of Th1 and Th2 type responses, since two distinct profiles of cytokine are the most reliable marker for distinction between these subsets of Th cells.

Cytokine-specific ELISAs for detecting IL-2, IL-4, IL-5, IL-6, IL-10 and IFN-γ are well-established and widely performed with a standard set of specific monoclonal antibodies (mAbs) (Table 1) (Okahashi *et al.*, 1996; Van Cott *et al.*, 1996). The following protocol is routinely used to detect cytokines in serum and external secretion samples, as well as in tissue culture supernatants.

1. Dilute capture antibody (Table 1) in phosphate buffered saline (PBS) and add 100 µl to the wells of a 96-well microtitre plate, Falcon Microtest III plates (Becton Dickinson, Oxnard, CA) or NUNC MaxiSorp (Nalge Nunc International, Naperville, IL). Incubate plates overnight at 4°C.
2. Remove solution from wells and block the remaining binding sites with PBS containing 1% bovine serum albumin (BSA) for 1 h at room temperature. Wash the plate three times by filling wells (200 µl/wash) with PBS and decanting the contents.
3. Generate standard curves using murine rIFN-γ, rIL-5, rIL-6 and rIL-10 (Genzyme Diagnostics, Cambridge, MA), rIL-2 (PharMingen, San Diego, CA) and rIL-4 (Endogen, Boston, MA). Prepare two-fold serial dilutions of recombinant cytokine standards and unknown samples diluted in PBS containing 0.05% Tween 20 (PBS-T) and 1% BSA. Add 100 µl/well IL-2 and IL-5 (diluted from 5–2000 pg ml^{-1}), IFN-γ, IL-4, IL-6 and IL-10 (diluted from 20–10 000 pg ml^{-1}). Prepare control wells for each cytokine standard by substituting a different standard as the only change. For example, for the IL-2 ELISA, use recombinant IL-4 as a background control. Incubate plate overnight at 4°C.
4. Wash plate six times with PBS-T and blot dry. Fully aspirate any remaining fluid from the wells by patting the bottom of the plate with dry absorbent paper.
5. Add 100 µl/well appropriate biotinylated capture mAb diluted in PBS-T with 1% BSA (Table 1). Incubate overnight at 4°C.
6. Wash plate six times with PBS-T, blot dry and add 100 µl/well peroxidase-labelled anti-biotin Ab (0.5 mg ml^{-1}; Vector Laboratories, Inc., Burlingame, CA) for 1 h at room temperature.
7. Wash plate six times with PBS, blot dry and develop with the chromogenic substrate, ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) (0.6 mg ml^{-1}) with 0.01% H_2O_2 (Moss, Inc., Pasadena, MD) for 90 min at room temperature. Read absorbance at 414 nm.
8. The ELISA assays are capable of detecting 5 pg ml^{-1} IL-2 and IL-5, 10–20 pg ml^{-1} IFN-γ, IL-4 and IL-10, and 60 pg ml^{-1} IL-6. If there are background problems associated with a particular biological fluid such as serum, then add 2% rat serum to the dilution buffer in step 3 to block non-specific binding sites of the detector mAb.
9. Calculate the concentrations of the unknown samples by reference to the linear portion of the standard curve.

Mouse-specific ELISPOT

ELISPOT protocol

The cytokine-specific ELISPOT is used to detect and quantitate the frequency of individual cytokine-secreting cells. The ELISPOT is performed using the same concept as the ELISA, with three key modifications: (1) the

Table 1. Reagents used for cytokine ELISA and ELISPOT assays to detect murine cytokines

		Monoclonal antibodies*			
		Coating (rat anti-mouse)		Detection (biotin–rat anti-mouse)	
Cytokine	Assay	Designation (clone)	Conc. (μg ml^{-1})	Designation (clone)	Conc. (μg ml^{-1})
IFN-γ	ELISA	R4-6A2	2.5	XMG1.2	0.3
	ELISPOT	R4-6A2	5.0	XMG1.2	0.1–2.5
IL-2	ELISA	JES6-1A12	2.5	JES6-5H4	0.4
	ELISPOT	JES6-1A12	5.0	JES6-5H4	0.2–2.5
IL-4	ELISA	BVD4-1D11	2.0	BVD6-24G2	0.2
	ELISPOT	BVD4-1D11	2.5	BVD6-24G2	0.1–2.5
IL-5	ELISA	TRFK-5	2.5	TRFK-4	4.0
	ELISPOT	TRFK-5	5.0	TRFK-4	0.2–2.5
IL-6	ELISA	MP5-20F3	2.0	MP5-32C11	0.5
	ELISPOT	MP5-20F3	5.0	MP5-32C11	0.5–2.5
IL-10	ELISA	JES5-2A5	2.0	JES5-16E3	0.3
	ELISPOT	JES5-2A5	5.0	JES5-16E3	0.2–2.5

96-well microtitre plate has a nitrocellulose base; (2) the unknown sample is a cell suspension rather than a biological fluid or tissue-culture supernatant; and (3) the concentration of detection mAbs is generally more concentrated. The following protocols were adapted to detect cytokine-secreting cells in single-cell suspensions from systemic sites (i.e. spleens) and mucosal sites (e.g. Peyer's patches, intestinal lamina regions and epithelium) (Taguchi *et al.*, 1990b; Fujihashi *et al.*, 1993b; Xu-Amano *et al.*, 1993; DiFabio *et al.*, 1994; Van Cott *et al.*, 1996).

1. Dilute capture mAb in PBS and add 100 μl to the wells of a nitrocellulose-backed microtitre plate (Millititer-HA, Millipore Corp., Bedford, MA) (Table 1). Place the plate in a humidified chamber, or carefully wrap it in saran wrap, and incubate overnight at 4°C.
2. Remove fluids from plate and block remaining binding sites with 100 μl/well culture medium (RPMI 1640 containing 5% FCS, 25 IU ml^{-1} penicillin, 25 μg ml^{-1} streptomycin and 80 μg ml^{-1} gentamicin) for 1 h at 37°C in a 5% CO_2 incubator.
3. Rinse the plate three times with PBS without Tween 20, and shake off excess fluids from plate.

4. Prepare five-fold dilutions of $CD4^+$ T cell suspensions prepared by flow cytometry sorting in culture medium starting at 10^6–10^7 cells/ml. Immediately add 100 µl/well of the cells to the capture mAb coated microtitre plate. Incubate the plate, with a plastic cover, containing $CD4^+$ T cells for 12–16 h at 37°C with 5% CO_2. The time required to purify $CD4^+$ T cells from freshly isolated cell suspensions significantly reduces the number of detectable cytokine-secreting cells. Thus, it is important to prepare the single-cell suspension in a prompt manner. For the assessment of cytokine production by antigen-specific $CD4^+$ T cells of mice, cells are purified from the different tissues and restimulated *in vitro* with the same antigen in the presence of irradiated feeder cells. After 1–6 days of antigen stimulation, $CD4^+$ T cells are harvested and immediately added to the capture mAb coated plates, as described above. In order to confirm the results obtained by ELISPOT assay, an aliquot of purified $CD4^+$ T cells can be subjected to cytokine-specific mRNA by RT-PCR (see pages 264–270).
5. Wash plates three times with PBS followed by three washes with PBS-T in order to remove the $CD4^+$ cells from individual wells. Before final aspiration, soak plates in PBS-T for 10 min to allow any remaining cells to detach from the plate. Add biotinylated detection mAb diluted in PBS-T to each well (Table 1). Place a plastic cover on the plate and incubate the plate overnight at 4°C.
6. Wash the plate six times with PBS-T. Remove excess liquid by removing the plastic covering around the base of the plate and by beating the plate over an absorbent towel. Place the plastic cover back on the plate and add peroxidase-labelled anti-biotin Ab (0.8 mg ml^{-1}) (Vector Laboratories, Inc.) diluted in PBS-T. Incubate at room temperature for 1 h.
7. Wash plates six times with PBS without Tween, as described in step 6. Develop spots with the substrate AEC (3-amino-9-ethylcarbazole) (0.3 ml ml^{-1}) (Moss Inc.).
8. Count the formation of red spots representing the corresponding cytokine producing $CD4^+$ T cells using a dissecting microscope (SZH Zoom Stereo Microscope System, Olympus, Lake Success, NY). Distinction of true spots from pseudo-spots should be done by comparing experimental wells with control wells. Controls should include:
   - coating with PBS instead of an anti-cytokine Ab;
   - coating with a different anti-cytokine Ab;
   - substituting a different biotinylated secondary Ab;
   - adding 50 µl ml^{-1} cycloheximide during the cell incubation period to inhibit *de novo* cytokine synthesis;
   - using unstimulated and concanavalin A (ConA) activated cells from unimmunized mice as negative and positive controls, respectively.

In addition, established and well-characterized Th1 or Th2 type $CD4^+$ T cell clones can be used as positive controls.

Analysis of antigen-specific Th1 and Th2 responses

The cytokine-specific ELISA and ELISPOT protocols have recently been used to distinguish and characterize recombinant (r)*Salmonella* and cholera toxin (CT) induced Th1 and Th2 cell responses (Xu-Amano *et al.*, 1993; Marinaro *et al.*, 1995; Van Cott *et al.*, 1996). Mice were orally immunized with two different forms of antigen-delivery system, including r*Salmonella* expressing fragment C (Tox C) of tetanus toxin or tetanus toxoid (TT) plus CT as mucosal adjuvant. Four to six weeks later, $CD4^+$ T cells were isolated from the Peyer's patches of each group and cultured with an optimal amount of soluble TT (1 $\mu g\ ml^{-1}$) and irradiated splenic adherent feeder cells (1×10^6 to $2\times10^6\ ml^{-1}$) from naive mice. After a 4-day incubation period, culture supernatants were harvested for the assessment of IFN-γ and IL-4 production as an indicator for Th1 and Th2 responses, respectively. IFN-γ (Th1 cytokine) and IL-4 (Th2 cytokine) specific ELISA revealed that orally administered r*Salmonella* Tox C induced IFN-γ production, whereas CT plus TT elicited IL-4 production by Peyer's patch $CD4^+$ T cells. In parallel, $CD4^+$ T cells were isolated from the culture for the enumeration of Th1 and Th2 cytokine-producing antigen-specific $CD4^+$ T cells by ELISPOT. Th1-type IFN-γ producing cells (about 760 per 10^6 $CD4^+$ T cells) were dominant in mice orally immunized with r*Salmonella* Tox C. In contrast, Th2-type IL-4 producing TT-specific $CD4^+$ cells (140 per 10^6 $CD4^+$ T cells) were induced in mice orally immunized with mucosal vaccine containing TT and CT (Fig. 1). Thus, the levels of secreted IFN-γ and IL-4 producing TT-specific Th cells. Using both Th1 and Th2 cytokine-

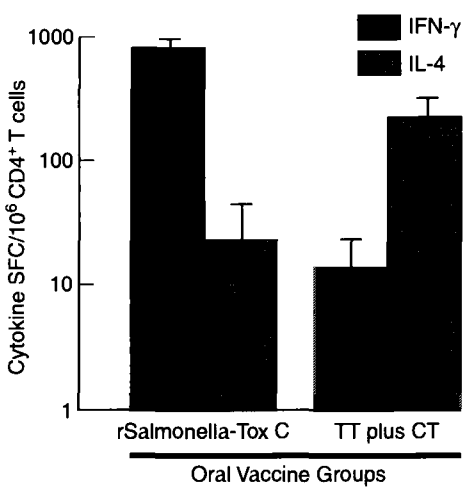


Figure 1. The frequency of Th1 and Th2 type cytokine spot-forming cells (SFCs) in TT-stimulated Peyer's patch $CD4^+$ T cells from mice orally immunized with r*Salmonella* Tox C, or CT plus TT. $CD4^+$ T cells were harvested from *in vitro* cultures following 4 days of incubation. Numbers of IFN-γ and IL-4 SFCs were examined by cytokine-specific ELISPOT. To determine the number of TT-induced IFN-γ and IL-4 SFCs, the number of SFCs in cultures without antigen (unstimulated) were subtracted from the number in cultures with antigen.

specific ELISA and ELISPOT assays, one can elucidate the type of Th1 and Th2 responses induced by different forms of vaccine at the level of both protein and cell, respectively.

Quantitative RT-PCR

An adaptation of RT-PCR technology in the area of immunology led to the detection of Th1 and Th2 cytokine-specific mRNA in different subsets of T cells. Furthermore, the recent development of quantitative RT-PCR allowed us to elucidate alterations in Th1 and Th2 cytokine specific mRNAs. Thus, increased or decreased levels of specific cytokine expression can be examined. For example, it is now possible to assess the molecular amounts of Th1 (e.g. IFN-γ) or Th2 (e.g. IL-4) cytokine-specific mRNA after *in vitro* stimulation of antigen-specific $CD4^+$ T cells from experimental animals mucosally and/or systemically immunized with different antigens or vaccines. Detailed protocols for Th1 and Th2 cytokine-specific quantitative RT-PCR, which is routinely performed in our laboratories, is described below.

Materials

RNA isolation

- TRIZOL (total RNA isolation reagent; Life Technologies).
- Chloroform.
- Isopropanol.
- 70% Ethanol.

Reverse transcription of RNA

- Oligo$(dT)_{12-18}$ primer (0.5 mg ml^{-1} in DEPC-water; Life Technologies).
- 100 mM Dithiothreitol (DTT) (Life Technologies).
- 5× Reverse transcription buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl and 15 mM $MgCl_2$) (Life Technologies).
- 10 mM dNTPs (Pharmacia).
- 20 U μl^{-1} RNase inhibitor (Promega).
- Super Script II (RNase H^- reverse transcriptase, 200 U ml^{-1}) (Life Technologies).
- 10 μg ml^{-1} RNase H (Life Technologies).
- Thermal cycler 9600 (Perkin Elmer).

All solutions and buffers are made with DEPC-treated water.

Polymerase chain reaction

- 10× Taq Buffer (Pharmacia).
- dNTP (2.5 mM each dATP, dTTP, dCTP, dGTP) (Pharmacia).

- 2500 U ml^{-1} Taq DNA polymerase (1:1 treatment of anti Taq antibody; Clontech) (Pharmacia).
- Amplification primers; 5.0 µM each 5′- and 3′-primers specific for cytokine of interest.
- Several concentrations of internal standard rDNA.

Capillary electrophoresis

- Capillary electrophoresis unit: P/ACE 5010 equipped with argon laser (Beckman Instruments).
- eCAP dsDNA 1000 Gel Buffer (Beckman Instruments).
- LIFluor EnhanCE (Beckman Instruments).
- 47 cm × 100 µm dsDNA 1000 coated capillary (Beckman Instruments).

Methods

Isolation of total RNA from mouse T cells (e.g. γδ and αβ T cells)

1. Isolate purified γδ and αβ T cells from different tissues (e.g. intestinal epithelium and spleen) by using a flow cytometry cell sorter (e.g. FACS) after the specific monoclonal antibody staining.
2. Wash isolated γδ and αβ T cells (~10^6) in PBS in a 1.5-ml Eppendorf tube.
3. Remove supernatant and add 0.5 ml TRIZOL. Lyse cells in TRIZOL by shaking thoroughly and incubate for 5 min at room temperature.
4. Add 0.1 ml chloroform, shake thoroughly and incubate at room temperature for 2–3 min.
5. Centrifuge the samples at 16 000*g* for 15 min at 4°C. Following centrifugation, the RNA is present in the colourless upper aqueous phase.
6. Carefully harvest the aqueous phase and transfer to a fresh tube.
7. Add 0.5 ml isopropanol, shake thoroughly and place at –80°C for at least 20 min.
8. Centrifuge the samples at 16 000*g* for 10 min at 4°C, and remove the supernatant.
9. Wash the RNA pellet once with 0.5 ml 70% ethanol. Mix the sample by vortexing and centrifuge at 16 000*g* for 5 min at 4°C.
10. Remove the supernatant and briefly dry (~5 min) the RNA pellet using the vacuum drier.
11. Dissolve the RNA in 10 µl DEPC-water.
12. Use 1 µl of the RNA samples to determine the A_{260} and A_{280} in order to calculate the RNA content and purity. One A_{260} unit equals 40 µg single-stranded RNA per millilitre. RNA samples having an A_{260}/A_{280} ratio of <1.8 are suitable for RT-PCR.

Reverse transcription of RNA

1. Add 1 µl Oligo(dT)$_{12-18}$ primer to 9 µl dissolved RNA sample. Incubate the mixture for 10 min at 70°C and place on ice for 1 min.
2. Prepare a master solution by adding the following: 4 µl reverse transcription buffer, 2 µl DTT, 2 µl dNTPs and 0.5 µl RNase inhibitor. Add this solution to the RNA sample and incubate the mixture for 5 min at 42°C.
3. Add 1 µl Super Script II and mix gently by repetitive pipetting. Incubate the mixture for 1 h at 42°C and then 15 min at 70°C to destroy reverse transcript activity.
4. Add 1 µl RNase H and incubate them for 30 min at 37°C. Store the solution at –20°C.

Construction of internal standard DNA for murine Th1/Th2 cytokines

For the quantitation of cytokine-specific message, recombinant DNA (rDNA) is constructed as internal standard. Internal standard is composed of three fragments including 5′ cytokine-specific primer, base fragment, and 3′ cytokine-specific primer and poly(dT)$_{16}$.

1. Synthesize three sets of forward and reverse oligo DNA primers using a DNA synthesizer (e.g. connected IL-5 and IL-6 primers are designed as the first primers; the second primers consisted of IL-4 and β-actin; the third primers contained IL-2 and IFN-γ). Attach poly(dT)$_{16}$ to the third reverse primer (e.g. IL-2 and IFN-γ) (Fig. 2).
2. Obtain an optimal length of spacer gene (about 10–50 bp length difference between the internal standard and the target PCR product is ideal).
3. Connect the first forward and reverse primers to the spacer gene by PCR. Mix 50 µl reaction mixture containing PCR buffer (16.6 mM $(NH_4)_2SO_4$, 50 mM 2-mercaptoethanol, 6.8 mM EDTA, 67 mM Tris-HCl, pH 8.8, 0.1 mg ml^{-1} BSA), 3 mM $MgCl_2$, 0.2 mM of each dNTP, 30 pmol of the first forward and reverse primer set for IL-5 and IL-6, 200 ng spacer gene and 2.5 U Taq DNA polymerase (Perkin-Elmer Cetus) and carry out 30 cycles of amplification: denature for 10 s at 94°C, anneal for 30 s at 59°C, extension for 45 s at 72°C; an additional extension for 5 min at 72°C after the last cycle.
4. Purify the PCR products with a Wizard PCR Preps DNA purification System (Promega).
5. Repeat the PCR procedures described in steps 3 and 4 twice more using the second (IL-4 and β-actin) and third (IFN-γ and IL-2) forward and reverse primers.
6. Insert the connected PCR product (IFN-γ, IL-2, IL-4, IL-5, IL-6, and β-actin) into pGEM-T Vector (Promega). Mix 1 µl T4 DNA Ligase 10× buffer, 1 µl pGEM-T vector, 1 µl 10× diluted PCR product, 1 µl

T4 DNA Ligase and 6 µl dH_2O, and incubate the mixture for 3 h at 15°C. Heat the reaction for 10 min at 70–72°C and allow to cool at 25°C.
7. Transform pGEM-T vector into *Escherichia coli* competent cells for selection of a positive colony.
8. Amplify the plasmid containing internal standard by incubation in 1 l LB broth overnight at 37°C.
9. Purify the plasmid DNA (internal standard) by using Wizard *Plus* Maxiprep (Promega).

PCR amplification of cDNA with internal control

(Wang *et al.*, 1989)

1. Prepare a series of dilutions of DNA internal standards (Fig. 3; IFN-γ).
2. Add 0.5 µl cDNA template (RNA 10 µg ml^{-1}) to the mixture of 2.5 µl 10× Taq buffer, 0.25 µl Taq DNA polymerase (1 : 1 treatment of anti-Taq antibody), 2 µl dNTP (2.5 mM each dCTP, dTTP, dGTP and dATP), 0.5 µl mixed primers and 0.5 µl diluted internal standard.
3. Carry out 28 cycles of PCR, including denaturing for 30 s at 94°C, annealing for 30 s at 60°C and an additional 7 min at 60°C as a final extension after the last cycle.

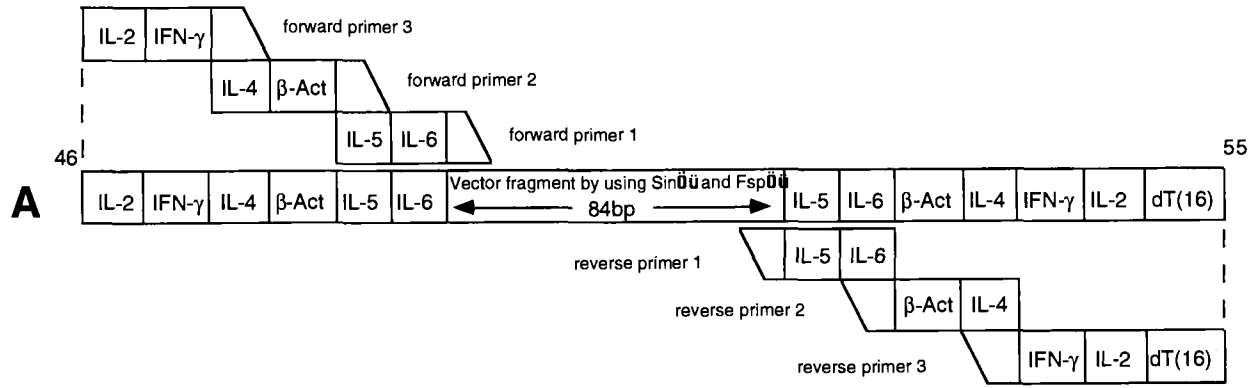
Capillary electrophoresis

(Schwartz and Ulfelder, 1992; Lu *et al.*, 1994; Rossomando *et al.*, 1994; Schwartz *et al.*, 1994; Fasco *et al.*, 1995)

1. Dissolve eCAP dsDNA 1000 Gel Buffer with 20 ml dH_2O by stirring overnight at room temperature, and add 8 µl LIFluor EnhanCE to the gel buffer before use.
2. Rinse and fill an empty capillary with gel buffer mixture.
3. Apply PCR products solution to the P/ACE 5010 with an injection time of 10 min. Perform capillary electrophoresis for 30 min in 9.4 kV for the actual analysis (Fig. 4).

Analysis of results

A plot for the ratio of the IFN-γ competitor to IFN-γ target peak area is calculated and illustrated (see Figs 3–5). Assuming that the reverse transcription reaction was 100% efficient, the intercept on the *x* axis from the regression line ($\gamma_2 = 0.995$) corresponding to an IFN-γ competitor to IFN-γ target ratio of one equals the quantity of RNA containing 1.0 pmol (the competitor concentration) of target sequence. Therefore, an amount of 3 ng RNA contained 1.0 pmol of IFN-γ target sequence. It was then indicated as 0.33 pmol IFN-γ/1 pg RNA.



Cytokines	5' Specific Primers	3' Specific Primers
IFN -γ	5'-TGAACGCTACAC- -ACTGCATCTTGG-3'	5'-CGACTCCTTTTC- -CGCTTCCTGAG-3'
IL-2	5'-ATGTACAGCATG- -CAGCTCGCATC-3'	5'-GGCTTGTTGAGA- -TGATGCTTTGACA-3'
IL-4	5'-ATGGGTCTCAAC- -CCCCAGCTAGT-3'	5'-GCTCTTTAGGCT- -TTCCAGGAAGTC-3'
IL-5	5'-ATGACTGTGCCTC- -TGTGCCTGGAGC-3'	5'-CTGTTTTTCCTGG- -AGTAAACTGGGG-3'
IL-6	5'-TGGAGTCACAGAA- -GGAGTGGCTAAG-3'	5'-TCTGACCACAGTG- -AGGAATGTCCAC-3'
β-Actin	5'-TGGAATCCTGTGG- -CATCCATGAAAC-3'	5'-TAAAACGCAGCTC- -AGTAACAGTCCG-3'

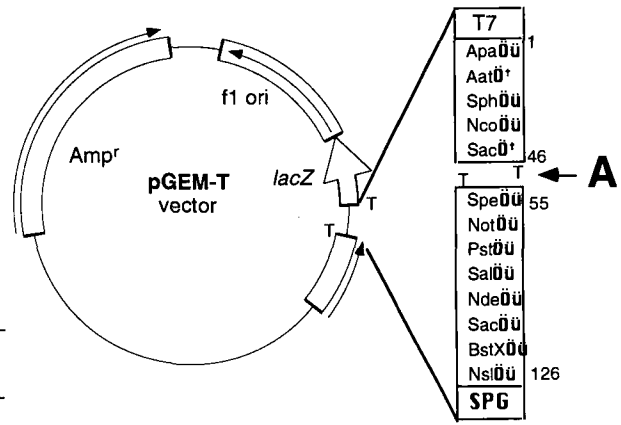


Figure 2. Design of a connected murine cytokine-specific recombinant RNA internal standard for quantitative RT-PCR.

Sample	Peak areas and points of plot obtained by CE-LIF			
	A	B	C	D
Target	311	238	182	118
[Amount of cDNA (ng) added]	(3)	(3)	(3)	(3)
Internal standard	57	152	254	422
[Amount of rDNA (pM) added]	(0.10)	(0.33)	(1.00)	(3.33)
Corrected ratio	0.15*	0.52	1.14	2.29

$$\text{Corrected ratio} = \frac{a \times c}{b}(d)^n;\ d = \frac{1+E_{In}}{1+E_{Ta}}$$

$$= \frac{57 \times \frac{460}{379}}{311}\left(\frac{1+0.50}{1+0.53}\right)^{20}$$

$$= 0.15^*$$

a = Correct peak area of the internal standard
b = Correct peak area of the target
c = Adjustment factor in order to adjust molecular size of internal standard to target molecule
d = PCR efficiency ratio between the internal standard (E_{In}) to the target (E_{Ta})
n = PCR cycles

Figure 3. An example for the quantitation of murine cytokine (IFN-γ) specific RT-PCR products.

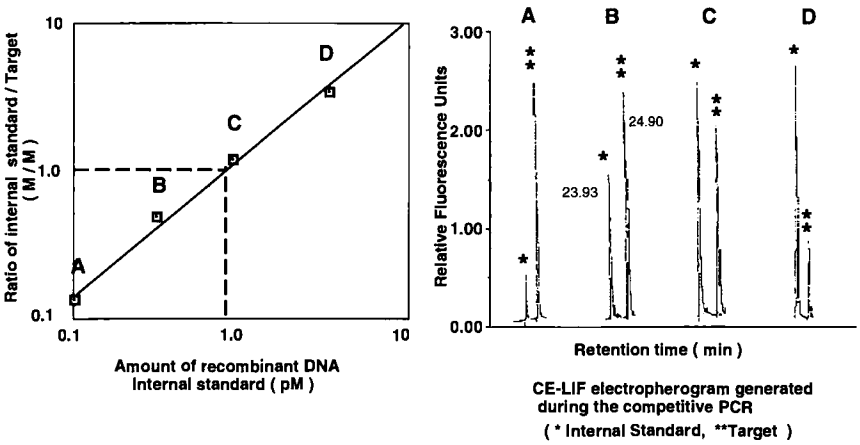


Figure 4. Peak areas and points of plot obtained by CE-LIF.

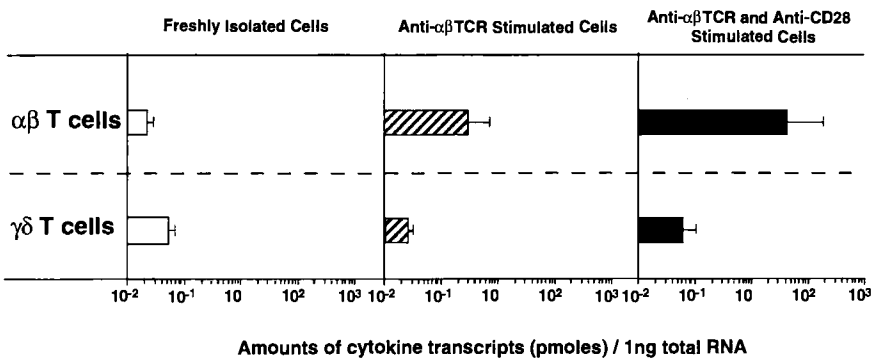


Figure 5. Level of IFN-γ specific mRNA in intraepithelial αβ and γδ T cells.

However, very high or low ratios obtained at highly disparate concentrations are not applicable to such analysis and/or calculation formula. Based on our experience of the analysis of numerous samples, we generally exclude ratios below 0.1 or above 30 from regression analyses.

Discussion

A decisive factor in understanding a quantitative RT-PCR assay is the inclusion of internal standards because of the reaction-to-reaction variation in amplification efficiency (Wang *et al.*, 1989). To derive the original from the amount of the amplified PCR product to the initial target concentration in the experimental sample is only possible if the amplification grade, and/or efficiency, is known. The amplification efficiency of the same target DNA might differ between reaction tubes. Therefore, this quantitative RT-PCR

method includes the following crucial advantages: (1) specific detection of the amplified samples; (2) rapid, non-radioactive test systems validated by an internal standard; and (3) quantitative evaluation of the assay, with calculation of the original amount in the sample *in vivo*.

It is essential to combine PCR with a sensitive secondary detection method in order to quantitate DNA sequences. To perform reliable quantitation, the PCR must be stopped between the 20th and 25th cycle, since it is then still within a linear amplification range. The capillary electrophoresis method, by using LIF, is a technique that can be used to detect low level PCR products (attomolar range). We demonstrate here a capillary electrophoresis method, using LIF detection that can analyse low levels (attomolar level of PCR products) of multiple DNA species generated during competitive or multiplex-competitive RT-PCR without additional treatment such as concentration of the samples.

◆◆◆◆◆◆ MEASURING NON-HUMAN PRIMATE CYTOKINES

Background

For the study of AIDS pathogenesis and vaccine development, the rhesus macaque/simian immunodeficiency virus (SIV) system is widely considered as the best experimental animal model available. It has been demonstrated that SIV can infect rhesus macaques via vaginal transmission, resulting in the development of an AIDS-like disease (Miller *et al.*, 1989). This system has been a useful model for the understanding of AIDS and related vaccine research, including the study of virus transmission via the mucosal barrier and the induction of virus-specific mucosal immunity. Although structural differences between SIV and HIV may provide complications for direct application of the vaccine results from the rhesus macaque/SIV model to humans, the model is useful for the development of a mucosal vaccine to prevent AIDS. For example, a combined schedule of intramuscular, oral and intratracheal immunizations provided protective immunity against vaginal challenge with SIV (Marx *et al.*, 1993). Furthermore, a combined mucosal immunization by vaginal followed by oral administration of virus-like particles carrying the SIV *gag* p27 and CT-B induced antigen-specific IgG and IgA responses in the reproductive tract (Lehner *et al.*, 1992). A sequence of rectal followed by oral immunization with the hybrid p27 antigen resulted in antigen-specific IgA and IgG responses in the rectal mucosa (Lehner *et al.*, 1993). It has recently been shown that targeted lymph node (TLN) immunization with a combined vaccine containing gp120 and p27 resulted in the generation of protective immunity against rectal challenge with SIV (Lehner *et al.*, 1994, 1996). In order to understand the mechanisms of the induction of SIV-specific immunity, it is essential to elucidate Th1 and Th2 cytokine responses. To this end, our group has adapted ELISA, ELISPOT and RT-PCR techniques for the assessment of rhesus macaque Th1 and Th2 responses (Imaoka *et al.*, 1998; Kawabata *et al.*, 1998; Kubota *et al.*, 1997).

Table 2. Reagents used for the characterization of Th1 and Th2 responses in rhesus macaques

		Coating antibody			Detection antibody			Recombinant cytokine	
Assay	Cytokine	Designation (clone)	Conc. (μg ml^{-1})	Source	Designation (clone)	Conc. (μg ml^{-1})	Source	Conc. (pg ml^{-1})	Source
ELISA	IFN-γ	B-B1	2.0	Biosource	Rabbit antisera	1.0	Endogen	5000-40	R&D systems
	IL-4	8D4-8	2.5	PharMingen	MP4-25D2	2.5	PharMingen	5000-20	R&D systems
ELISPOT	IL-10	JES3-9D7	5.0	PharMingen	JES3-12G8	2.0	PharMingen	5000-20	PharMingen

Non-human Primate-specific ELISA

ELISA protocol

In order to measure rhesus macaque-specific cytokine, we have screened and tested the cross-reactivity of human cytokine-specific reagents with rhesus macaque. Based on our screening, the appropriate anti-human IFN-γ, IL-4, IL-5 and IL-10 monoclonal and polyclonal antibodies are selected for the detection of rhesus macaque cytokines (Table 2). When the specificity and sensitivity of the assay were considered, it was found that a combination of two different clones of monoclonal antibodies specific for the same cytokine as capture and detection reagent was the best for detection of Th1 or Th2 cytokines. However, if only one monoclonal antibody is available, this antibody can be used as capture antibody and an additional polyclonal antibody is used as detection antibody. Recombinant human cytokines were used as standards for rhesus cytokine-specific ELISA.

Microwell 96-well microplates (MaxiSorp, Nalge Nunc International) were coated with 100 µl capture anti-cytokine antibodies diluted in PBS (Table 2) and incubated overnight at 4°C. The wells were blocked with PBS containing 3% BSA at room temperature for 2 h. The serially two-fold diluted culture supernatants from antigen, anti-CD3- or mitogen-stimulated rhesus lymphocytes and standard recombinant human cytokines were added to duplicate wells and incubated overnight at 4°C. The wells were washed with PBS containing 0.05% Tween 2 (PBS-T), and incubated with the appropriate biotinylated anti-cytokine antibodies diluted in PBS-T overnight at 4°C. For the detection of a Th1 cytokine (e.g. IFN-γ), monoclonal antibody (B-B1, Biosource) was used to capture antibody (Table 2). Biotinylated anti-human IFN-γ (rabbit antisera, Endogen) was prepared from polyclonal anti-human IFN-γ using sulphosuccinimidyl-6-(biotinamido)hexanoate, as described elsewhere (Merurkar *et al.*, 1984). For the detection of IL-4 and IL-10, each purified mAb (8D4-8 for IL-4 and JES3-9D7 for IL-10) was used as capture antibody. In addition, biotinylated antibodies obtained from the manufacturer were applied as detection antibodies (Table 2). The wells were washed with PBS-T, incubated with peroxidase-labelled anti-biotin antibody (0.25 µg ml^{-1}) (Vector Laboratories, Burlingame, CA) overnight at 4°C and developed with ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (0.6 mg ml^{-1}) (SIGMA, St Louis, MO) containing 0.01% H_2O_2. The plates were read at an optimal density of 415 nm using a microplate reader (model EL 312e, Bio-Tek Instruments, Winooski, VT). Standard curves were generated with known amounts of corresponding recombinant human cytokines placed in each plate. The levels of specific cytokine in the culture supernatants were interpolated on standard curves generated using a Macintosh computer program with four-parameter logistic algorithms.

Detection of SIV p55 stimulated rhesus CD4$^+$ T cells produced Th1 and Th2 cytokines

The PBMCs isolated from macaques given the combined oral vaccine containing $p55^{gag}$ (p55) (1 mg) plus CT (50 μg) were cultured either in the presence or absence of p55 (5 μg ml^{-1}) at a cell density of 1×10^6 cells per millilitre in complete medium in six-well flat-bottomed culture plates (Corning, New York) in order to characterize the Th1 and Th2 phenotype of p55-specific CD4$^+$ T cells (Kubota *et al.*, 1997). The culture supernatants were harvested from p55-stimulated or -unstimulated PBMCs 4 days after incubation, and the levels of Th1 and Th2 cytokines in culture supernatants were determined by a modified ELISA (DiFabio *et al.*, 1994; Van Cott, 1996; Kubota *et al.*, 1997). Th1 and Th2 cytokine production by antigen-specific cells were obtained by subtracting cytokine levels in cells cultured without antigen from the cytokine concentration in culture supernatants of p55-stimulated cells.

Higher levels of IFN-γ and IL-10, as Th1 and Th2 cytokines, respectively, were always detected in the culture supernatants harvested from *in vitro* p55-stimulated PBMCs when compared with unstimulated PBMCs (Table 3) (Kubota *et al.*, 1997). In contrast to IFN-γ and IL-10, IL-4 was not detected at any time during the experiment. Although we could determine antigen-induced Th1 and Th2 cytokine production by ELISA, this assay system may not reflect the exact Th1 and Th2 cytokine responses, since culture supernatants in this experimental system may contain cytokines produced by non CD4$^+$ T-cell fractions. In order to overcome this, purified CD4$^+$ T cells from PBMC of orally immunized macaques were cultured with APCs either in the presence or absence of p55; however, it is rather difficult to obtain sufficient numbers of purified CD4$^+$ T cells from the PBMCs of rhesus macaques. Thus, the macaque system has additional limitations for analysis of *in vitro* cytokine responses. Th1 and Th2 cytokine-specific ELISPOT assay provides an advantage for the characterization of Th1 and Th2 cytokine profiles mediated by CD4$^+$ T cells. The following section describes the general protocol for primate cytokine-specific ELISPOT assay and an example of actual experiments and results obtained from an SIV oral immunization study using rhesus macaque monkeys.

Table 3. The levels of increased Th1 and Th2 cytokine responses by p27-specific CD4$^+$ Th cells isolated from TLN immunized primates*

Cytokine	No. of cytokine-producing cells/10^4 cells	No. of molecules/1 ng total RNA
IFN-γ	0	< 10.0
IL-4	15.3 ± 4.1	56.3 ± 0.5

* Values are differences between the values before (day 0) and after (day 63) immunization.

Non-human Primate-specific ELISPOT Assay

ELISPOT protocol

The cytokine-specific ELISPOT assays for the detection of monkey IFN-γ and IL-4 were established by a modification of our protocols for murine Th1 and Th2 cytokine-specific ELISPOT assay (Taguchi *et al.*, 1990b; Fujihashi *et al.*, 1993b; Xu-Amano *et al.*, 1993; DiFabio *et al.*, 1994; Van Cott, 1996; Kawabata *et al.*, 1998; Kubota *et al.*, 1997). A variety of control wells were employed in order to ensure specificity of the formation of cytokine-specific spot-forming cells (SFCs), which include: (1) coating wells with PBS instead of cytokine specific capture antibodies; (2) adding 100 μg ml^{-1} cycloheximide during the cell incubation period in order to inhibit *de novo* cytokine synthesis; and (3) using $CD4^+$ T cells from unstimulated and ConA-activated cells from the same monkeys as negative and positive controls, respectively. The optimal concentrations of coating and biotinylated detection antibodies used in this study are listed in Table 2.

1. For all assays, 96-well nitrocellulose-based microtitre plates (Millitier-HA, Millipore Corp.) were coated overnight at 4°C with 100 μl of the appropriate concentration of anti-cytokine antibody diluted in PBS (Table 2) (pH 7.2–7.4) (Kubota *et al.*, 1997). Alternatively, antibodies can also be diluted in carbonate buffer (pH 9.6) or borate buffer (pH 8.4). The plates must be kept sterile until the end of the cell incubation.
2. Unbound coating capture antibodies are discarded and the plates are rinsed three times with sterile PBS. The wells were blocked using complete medium (RPMI 1640 containing 10% fetal calf serum (FCS), 25 IU ml^{-1} penicillin, 25 μg ml^{-1} streptomycin and 80 μg ml^{-1} gentamicin) for 2 h at 37°C. In some cases, serum of the host animal, which was used for the generation of detection antibody, is also added to the blocking medium in order to reduce the background.
3. Different concentrations of $CD4^+$ T cells (100 μl) were added to individual wells precoated with the respective capture antibody and incubated for 20 h at 37°C in a humidified, 5% CO_2 incubator. Five-fold cell dilutions starting at 1×10^5 or 1×10^6 cells/well are recommended. Each experimental condition was done in duplicate or triplicate, in order to ensure consistency of the results.
4. Cells were discarded and the plates washed extensively with PBS and then PBS-T to remove the adherent cells, since residual cells or cell membranes on the nitrocellulose will cause pseudo-spots. Furthermore, in order to remove excess liquid, the upper plate must be detached from the bottom frame and placed on a dry absorbent paper towel.
5. Appropriate concentrations of biotinylated anti-cytokine antibodies in PBS-T were added (100 μl/well) (Table 2) and the plates were

incubated overnight at 4°C. Since peroxidase conjugated goat anti-biotin antibody is used in the next step, in order to increase the sensitivity for the detection of respective cytokine specific spots, biotin conjugated secondary antibodies have to be diluted in PBS-T containing 5% goat serum.

6. The plates were washed three times with PBS-T and the upper plate must be detached from the bottom frame and placed with the absorbent material to remove residual buffer from the well. Following incubation with peroxidase-labelled anti-biotin antibody (Vector Laboratories Inc.) – overnight at 4°C, 4 h at room temperature or 2 h at 37°C – plates are washed 3 times with PBS and dried.
7. Cytokine-specific SFCs were visualized by adding 100 µl of the chromogenic substrate, AEC (3-amino-9-ethylcarbazole) solution (0.3 mg ml^{-1}) (Moss Inc.). The number of SFCs was counted using a dissecting microscope (SZH Zoom Stereo Microscope System, Olympus, Lake Success, NY).

The frequencies of antigen-specific Th1 and Th2 cytokine producing $CD4^+$ T cells from mucosally immunized rhesus macaques

The PBMCs isolated from vaccinated rhesus macaques are incubated with the antigen used for mucosal immunization (e.g. p55) as described above (see page 274). Following 4 days of incubation, $CD4^+$, $CD8^-$ T cells were isolated from p55-stimulated and/or unstimulated PBMC cultures by flow cytometry (FACStar^{Plus}, Becton Dickinson, San Jose, CA) using FITC-conjugated anti-CD4 (Leu-3a) monoclonal antibody (Becton Dickinson) and PE-conjugated anti-CD8 monoclonal antibody (Exalpha, Boston, MA), which have been shown to cross-react with rhesus T cells (Kawabata *et al.*, 1998; Kubota *et al.*, 1997). Purified $CD4^+$, $CD8^-$ T cells were then subjected to cytokine-specific ELISPOT assay. The numbers of SFCs formed in wells containing unstimulated $CD4^+$ T cells were subtracted from the numbers of SFCs developed in wells containing p55-stimulated $CD4^+$ T cells. When numbers of IFN-γ producing $CD4^+$ T cells were enumerated, high numbers of SFCs were generally noted in PBMCs from orally immunized macaques with p55 and CT as mucosal adjuvant (Fig. 6) (Kubota *et al.*, 1997). In contrast, the frequency of IL-4 SFCs was low in p55-stimulated PBMCs obtained from these immunized macaques (Fig. 6) (Kubota *et al.*, 1997). As controls, $CD4^+$ T cells from a macaque orally immunized with p55 only were subjected to Th1 and Th2 analysis. This monkey possessed low levels of antigen-specific antibody responses when compared with the experimental group of macaques orally immunized with p55 and CT. Furthermore, neither IFN-γ nor IL-4 producing cells were noted in the macaque receiving antigen only. Taken together, these findings suggested that oral immunization of non-human primates with a mucosal vaccine containing SIV p55 and CT induced both antigen-specific IFN-γ producing Th1 and select Th2 cytokines (e.g. IL-6 and IL-10, but not IL-4) secreting $CD4^+$ T cells (Kubota *et al.*, 1997), previously termed 'level-2 Th2 cells'

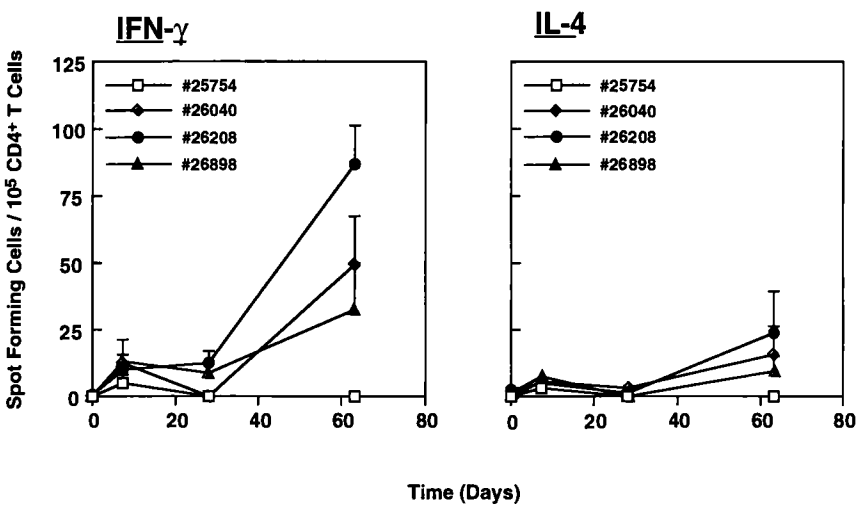


Figure 6. Analysis of Th1 and Th2 cytokine-producing $CD4^+$ T cells in orally immunized rhesus macaques. $CD4^+$ T cells from SIV-antigen stimulated PBMCs *in vitro* were purified by flow cytometry and cells were then subjected to IFN-γ- and IL-4-specific ELISPOT assays.

which preferentially produce selective cytokine for the induction of IgA responses (examples of IgA enhancing cytokines) (Van Cott *et al.*, 1996).

Quantitative RT-PCR for Non-human Primates

Selection of rhesus macaque-specific cytokine primers

The nucleotide sequence of the characterized rhesus macaque cytokine genes showed 93–99% homology to the published sequences of the equivalent human cytokine genes (Villinger *et al.*, 1995). Two approaches were taken in order to prepare a panel of Th1 and Th2 cytokine-specific primers for rhesus macaques. As a first approach, different human cytokine-specific primers were screened and tested for the detection of rhesus macaque cytokine mRNA expression. The mRNAs for IL-2 and IL-6 were successfully amplified by using human IL-2- and IL-6-specific primer sets when ConA stimulated PBMCs isolated from rhesus macaques were subjected to RT-PCR analysis. On the other hand, the use of human IFN-γ- and IL-4-specific primer sets failed to amplify IFN-γ and IL-4 messages in rhesus macaques. To overcome this problem, we aligned and compared human and mouse nucleotide and amino acid cDNA sequences, and identified the maximum homologous region. From this procedure, we selected several candidate sequences for monkey cytokine-specific primers. These primers were then screened and tested using ConA or PHA stimulated monkey PBMCs. For example, when the human and mouse IL-4 coding regions deduced from the nucleotide sequences are compared, the regions of the human IL-4 coding sequence from amino acid positions 1–90 and 129–149 share about 50% homology at the amino

acid level with the corresponding regions of the mouse IL-4 coding sequence (Fig. 7). The 5′ and 3′ untranslated regions of these human and mouse clones share about 70% DNA sequence homology. The region of human IL-4 covered by amino acid positions 91–128 shares very little homology with the corresponding region of mouse IL-4 at either the amino acid or nucleotide sequence levels. Thus, one pair of candidate primers was selected from between amino acid positions 1 and 90. The other possible candidate for monkey IL-4 specific primers was between amino acid positions 1 and 90 for the upper primer, and amino acid positions 129 and 149 for the lower primer (Fig. 7).

By employing this strategy, we successfully amplified the rhesus macaque IL-4 cDNA, which was reverse transcribed from mRNA. Thus, the primer sequences for the macaque (5′-primer, 5′-GTC CAC GGA CAC AAG TGC GAT-3′; 3′-primer, 5′-CAT GAT CGT CTT TAG CCT TTC-3′) was useful for the detection of rhesus macaque IL-4-specific message. Similar approaches were employed to construct rhesus macaque IFN-γ-specific primers (Fig. 8).

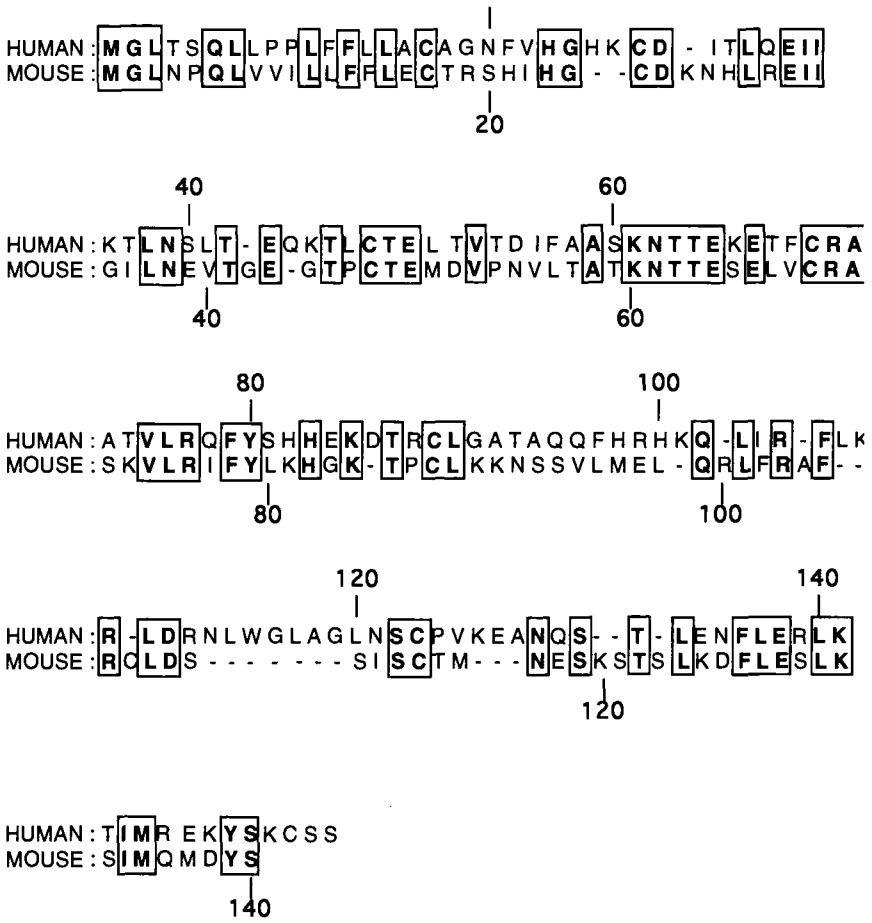
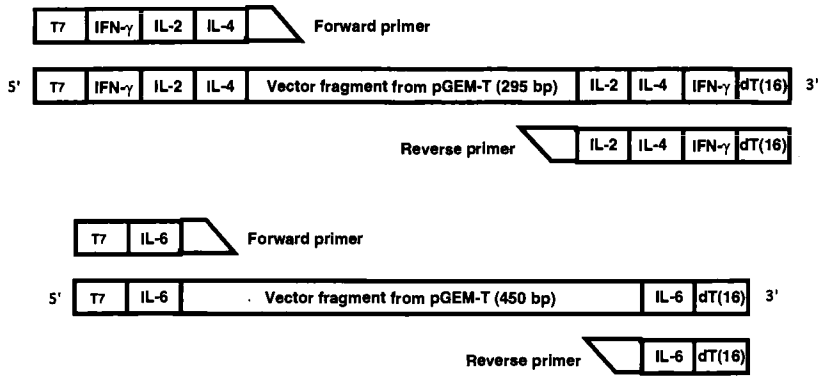


Figure 7. Alignment and comparison of human and mouse amino acid and cDNA sequences.



Cytokines	5' Specific primers	3' Specific primers
mon-IFN-γ	5'-CAg CTC TgC ATT gTT TTg ggT-3'	5'-CAT CTg ACT CCT TTT TCg CTT-3'
hu-IL-2	5'-ATg TAC Agg ATg CAA CTC CTg TCT T-3'	5'-gTT AgT gTT gAg ATg ATg CTT TgA C-3
mon-IL-4	5'-gTC CAC ggA CAC AAg TgC gAT-3'	5'-CAT gAT CgT CTT TAg CTT TTC-3'
hu-IL-6	5'-ATg AAC TCC TTC TCC ACA AgC gC-3'	5'-gAA gAg CCC TCA ggC Tgg ACT g-3'

Figure 8. Design of internal standard and primers specific for primate Th1 and Th2 cytokines. PCR conditions: denaturing at 94°C for 1 min; annealing at 60°C for 1 min; 35 cycles.

Quantitative RT-PCR for non-human primate cytokines

Recombinant RNA for the internal standard (rcRNA-IS) specific for each Th1 and Th2 cytokine was constructed in order to quantitate rhesus macaque-specific cytokine mRNA messages (Kawabata *et al.*, 1998). The detail of this procedure is given above (see pages 264–270) (Siebert and Larrick, 1992; Hiroi *et al.*, 1995; Fujihashi *et al.*, 1996).

Briefly, two spacer genes (185 and 253 bp) from pGEM-T vector (Promega) were obtained by digestion with the restriction enzyme *Rma* I and *Ssp* I (Promega), respectively (Fig. 8). The forward primer consisted of T7 promotor, the target mRNA specific 5'-primer and the 5'-primer for the spacer gene. The reverse primer contained sequences for poly(dT), 3' cytokine-specific primer and 3'-primer for the space gene. The internal standards were then constructed by PCR using the above primers. These PCR products were used as dsDNA internal standard (dsDNA-IS). The dsDNA-IS was then transcribed into RNA by the T7 promotor using the Riboprobe Gemini II *in vitro* transcription system (Promega). The rcRNA-ISs were subsequently treated with RNase free DNase to remove the DNA templates. For the quantitative competitive RT-PCR, amplification of sample RNA and rcRNA-IS was performed in the same tube. The amount of the PCR products was then measured by capillary electrophoresis using Gold-P/ACE System 5000 (Beckman Instruments, Fullerton, CA) (Srinivasan *et al.*, 1993; Lu *et al.*, 1994; Yamamoto *et al.*, 1996). In general, using this system we could detect 1 fmol per millilitre of PCR products.

Thus, the equivalent target gene mRNA level present in the RNA sample was determined from a known amount of rcRNA-IS concentration.

Correlation between quantitative RT-PCR and ELISPOT assay for the assessment of rhesus macaque Th1 and Th2 responses

In order to understand precisely the induction of antigen-specific Th1 and Th2 cells following mucosal and systemic immunization (e.g. oral, nasal and TLN) immunization of rhesus macaques, a combination of cytokine-specific ELISPOT and quantitative RT-PCR provided response patterns of Th1 and Th2 cells at cell and mRNA levels. These two systems should complement each other in order to understand the nature of Th1 and Th2 type antigen-specific $CD4^+$ T cell responses in these immunized macaques. An example of our recent results obtained by ELISPOT and RT-PCR for TLN immunized macaques is discussed below.

To elucidate the cytokine profiles at the mRNA level in rhesus macaques immunized with SIV antigen (e.g. gp120 and/or p27) using a TLN immunization procedure, RNA was isolated from an aliquot of p27-stimulated $CD4^+$ T cells sampled before (day 0) and after (day 63) immunization and subjected to quantitative RT-PCR (Kawabata *et al.*, 1997). In addition, another aliquot of $CD4^+$ T cells from pre- and post-immunization groups was also examined, to determine the number of Th1 and Th2 cytokine-producing cells by ELISPOT assay. In order specifically to demonstrate quantitative changes in Th1 and/or Th2 cytokine-specific mRNA expression, molecular amounts of the corresponding cytokine-specific mRNA of pre-immunization sample were subtracted from mRNA of the post-immunization group (Kawabata *et al.*, 1998). The number of corresponding cytokine-producing cells was also calculated in the same manner. When macaques were examined for levels of Th1 (IFN-γ) and Th2 (IL-4) mRNA by quantitative RT-PCR, no major increases in Th1-type cytokine, such as IFN-γ was noted in antigen-specific $CD4^+$ T cells obtained after immunization (Table 3). On the other hand, Th2 cytokine (IL-4) was increased following TLN vaccination with SIV antigen (56.3 molecules per 1 ng total RNA), while levels of IL-4 specific mRNA was less than 10 molecules of total RNA in control $CD4^+$ T cells. Identical patterns of cytokine profiles were also seen in results obtained by ELISPOT assay (Table 3). Thus, increased numbers (10–25 SFCs/10^4 $CD4^+$ T cells) of IL-4 producing cells were detected after TLN immunization. In contrast, although IFN-γ secreting cells were also seen in post-TLN-immunized macaques, frequency of these cells was decreased when compared with numbers of IFN-γ producing cells in the pre-immunization group. These results showed a correlation between two different cytokine-specific assays, which represent the level of mRNA and the frequencies of cytokine-producing cells. As a limited array of Th1 and Th2 cytokine-specific reagents is currently available for rhesus macaques, it would be useful to use at least two sets of Th1 and Th2 assays from ELISA, ELISPOT and RT-PCR.

◆◆◆◆◆◆ SUMMARY

In this chapter we have described the protocols for Th1 and Th2 cytokine-specific ELISA, ELISPOT and quantitative RT-PCR assays for both the mouse and non-human primates. These three assay systems allow detection of different stages of cytokine production, including secreted cytokine, cytokine-producing cells and cytokine-specific mRNA. Although each assay has unique advantages for the detection and quantitation of Th1 and Th2 cytokines at three distinct levels (e.g. protein, mRNA and cell) in these animal models, the use of individual assays in a separate manner may often not be sufficient for a thorough and accurate determination of the profile of Th1 and Th2 cytokine expression. For instance, when the levels of cytokine-specific mRNA are measured by quantitative RT-PCR only, the information concerning the biological activity of the analysed cytokine is unknown. Furthermore, examination of secreted cytokines in culture supernatants by ELISA may only detect residual cytokines produced by $CD4^+$ T cells *in vitro*, since it is possible and even likely that secreted cytokines may be immediately taken up by neighbouring cells in the culture. To this end, it is best to perform at least two different cytokine assays to confirm the results describing the profile of Th1 and Th2 cytokine responses induced by mucosal and/or systemic immunization. An ideal situation would be to use all three assays for the elucidation of Th1 and Th2 responses.

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2.5 Isolation of and Measuring the Function of Professional Phagocytes: Murine Macrophages

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CONTENTS

Introduction
Isolation of macrophages
Culture of macrophages
Measuring macrophage function
Conclusion

List of Abbreviations

AcLDL	acetylated low density lipoprotein
BCG	Bacille Calmette-Guérin
BgPM	Biogel-elicited peritoneal macrophage
BMDM	bone marrow derived macrophage
BP	bacteriologic plastic
BSA	bovine serum albumin
CD	cluster of differentiation
cfu	colony-forming units
CR3	complement receptor type 3
DiI	1,1′-dioctadecyl-1-3,3,3′,3′tetramethylindocarbocyanine perchlorate
DMEM	Dulbecco's modified Eagle's medium
ECM	extracellular matrix
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene bis (oxyethylenenitrilo) tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMEM	Eagle's minimum essential medium
FACS	fluorescence activated cell sorting/scanning
FcR	receptor for Fc portion of immunoglobulin
FCS	fetal calf serum

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FITC	fluorescein isothiocyanate
HBSS	Hank's buffered saline solution
HEPES	*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]
HRP	horseradish peroxidase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
LPS	lipopolysaccharide
LTA	lipoteichoic acid
mAb	monoclonal antibody
M-CSF	macrophage colony-stimulating factor
MEM	modified Eagle's medium
MHC	major histocompatibility complex
MMR	macrophage mannose receptor
NMMA	*N*-monomethyl arginine
NMS	normal mouse serum
NO	nitric oxide
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PMA	12-myristate 13-acetate
PMN	polymorphonuclear cell (neutrophil)
Poly G	Polyguanylic acid
RBMM	resident bone marrow macrophage
RPM	resident peritoneal macrophage
RPMI	RPMI 1640 medium
SD	standard deviation
SR-A	macrophage scavenger receptor class A
SRKO	scavenger receptor class A knockout mouse
TBAC	Tris buffered ammonium chloride
TCP	tissue culture plastic
TNF	tumour necrosis factor
TPM	thioglycollate-elicited peritoneal macrophage

◆◆◆◆◆◆ INTRODUCTION

Professional phagocytes can be divided into polymorphonuclear leukocytes (PMNs) and macrophages (Gordon *et al.*, 1988, 1992; Van Furth and Cohn, 1968). In this chapter methods for measuring functions of murine macrophages *in vitro* are discussed. Those seeking information on neutrophils are referred to methods reported elsewhere (Leijh *et al.*, 1986). A wide range of techniques is available for assessing the role of macrophages *in vivo* (Rooijen and Sanders, 1994), and some of these are discussed elsewhere in this volume (chapters by Ehlers and Seitzer, and by Roberts *et al.*).

Macrophages play a key role in host defence and carry out a range of functions, including phagocytosis and killing of micro-organisms, cytotoxic activities and secretion of a wide range of bioactive factors (Leijh *et al.*, 1986). Macrophages participate in antigen presentation to primed T lymphocytes

and also influence the resulting immune response by releasing cytokines (Stein and Gordon, 1991). Macrophage effector functions, combined with complement-specific antibodies and stimulated B and T cells are vital to resistance to opportunistic and virulent micro-organisms, as demonstrated by the lethal complications of prolonged, severe immunodeficiency. In addition, macrophages play a role in recruiting other leukocytes to sites of inflammation through release of locally active chemokines, e.g. IL-8 and Rantes (Adams and Lloyd, 1997; Sarafi *et al.*, 1997). There are well-established methods for studying the locomotion and chemotaxis of professional phagocytes *in vitro* (Wilkinson, 1986).

Macrophage plasma membrane receptors, biosynthesis and metabolic responses vary greatly during cell maturation, migration and phenotypic modulation. This cellular heterogeneity must be considered when investigating the interaction between macrophages and micro-organisms. Indeed, this variety of macrophage phenotype has provided obstacles to the use of macrophages as targets of drug delivery (Gordon and Rabinowitz, 1989).

This chapter provides a practical guide not only for the isolation of murine monocyte/macrophage populations, but also for a range of assays enabling the measurement of a number of macrophage functions. An explanation of abbreviations is provided at the beginning of the chapter and a list of commercial suppliers at the end of the chapter.

◆◆◆◆◆◆ ISOLATION OF MACROPHAGES

Peritoneal Macrophages

A convenient source of primary macrophages is the peritoneal cavity of the mouse. This site provides a high yield of cells from which macrophages can be purified through an adhesion step. In addition, the investigator can obtain macrophages of differing phenotypes and activation status depending on the stimulus used to recruit cells to the site. In broad terms, peritoneal macrophages can be divided phenotypically into resident, elicited and activated cells according to stimulus. These macrophages are collected following peritoneal lavage, as outlined below.

Resident peritoneal macrophages

Mice are killed by carbon dioxide inhalation and then pinned down with their ventral surface uppermost. The skin is wetted with 70% ethanol in water and, using fine scissors, a small lateral cut is made in the skin over the abdomen. Care must be taken not to cut into the body wall at this stage. The skin is pulled back from the incision to reveal the shiny surface of the body wall. This area must remain sterile during the entire procedure. Then, using a 21-gauge needle, approximately 10 ml of sterile saline is injected slowly into the peritoneal cavity. The needle should be inserted bevel uppermost into the caudal half of the abdomen and care

taken not to penetrate any organs. As the needle is removed there may be some loss of saline; however, omental fat will usually block further leakage. The pins are removed from the forelegs of the mouse and the entire body shaken gently for approximately 10 s. Using a 19-gauge needle attached to a 10-ml syringe, the saline is slowly removed from the peritoneal cavity. The needle should be inserted bevel down into the cranial half of the cavity to avoid fat blocking the needle during aspiration. Following removal of the saline from the cavity the needle is withdrawn, and the cell suspension can then be stored in a tube placed on ice until required.

Thioglycollate broth elicited cells

The use of intraperitoneal injection of sterile inflammatory reagents provides a useful method of isolating large numbers of macrophages for functional assays *in vitro*. Thioglycollate-elicited peritoneal macrophages (TPMs) can be recruited to the peritoneal cavity by intraperitoneal injection of 1 ml Brewer's complete thioglycollate broth (Difco Laboratories) (Johnson *et al.*, 1978). Cells are harvested as outlined for resident peritoneal macrophages (RPMs) above, 4–5 days following injection. TPMs ingest large amounts of the inflammatory agent (agar) but retain active endocytic and phagocytic function on isolation. The use of proteose peptone as a stimulant produces cells with a phenotype similar to that of TPMs, but with fewer phagocytic vacuoles, and the cell yields are lower. Thioglycollate broth often contains low levels of lipopolysaccharide (LPS) (0.5 ng ml^{-1}), which should be considered since this may affect macrophage responsiveness in subsequent assays.

Biogel polyacrylamide bead elicited cells

The need to obtain large numbers of elicited macrophages has resulted in the testing of other inflammatory stimuli. The first reported use of polyacrylamide beads for this purpose was by Fauve *et al.* (1983). They injected beads into subcutaneous pouches created in the dorsal skin of mice. In this model, 10^7 phagocytic cells (60% macrophages, 40% PMNs) could be recovered from the resulting 'granuloma'. In our laboratory, Biogel beads have been used successfully to elicit a high yield of peritoneal macrophages (10^7 cells per mouse). The most suitable size of bead is P100 (hydrated size 45–90 nm), which macrophages are unable to ingest or extracellularly digest.

Method

Biogel polyacylamide beads (Biogel P-100 (fine), Bio-Rad Laboratories) are washed in phosphate buffered saline (PBS) by repeated centrifugation, and autoclaved before use. Following peritoneal injection with 1 ml of a 2% v/v suspension, macrophages can be harvested from day 4 or 5 at yields of 1×10^7 macrophages per mouse. To purify the macrophages,

cells can be routinely plated in medium at 3×10^5 per well in a 24-well tissue-culture plate. After incubation for 60–90 min at 37°C, the non-adherent cells can be removed by washing the wells five times with PBS. Under these conditions, the adherent monolayers consist of more than 90% macrophages, and viability is usually greater than 97% by phase contrast microscopy and Trypan Blue exclusion.

Biogel-elicited peritoneal macrophages (BgPMs) have a number of features that distinguish them phenotypically from the more familiar TPMs. For example, following incubation overnight in serum-containing medium on tissue culture plastic, 50% of BgPMs will have become completely non-adherent and the remainder will have rounded up. In contrast, TPMs will remain flattened and tightly adherent to the substratum (M. Stein, unpublished observations). In addition, the culture medium selected and the presence of serum has profound effects on the degree of spreading of BgPMs (Fig. 1). Some differences in phenotype between these elicited and other macrophage populations are listed in Table 1.

One of the most useful markers of murine macrophages is defined by the monoclonal antibody (mAb) F4/80, which recognizes a 160-kDa glycoprotein on the surface of most mouse macrophage populations (Austyn and Gordon, 1981; McKnight *et al.*, 1996). No function has yet been ascribed to F4/80, although its expression is known to be down-regulated by γ-interferon (IFN-γ) and in response to Bacille Calmette-Guérin (BCG) infection (Ezekowitz *et al.*, 1981; Ezekowitz and Gordon, 1982) (see Table 1).

The FA11 antibody recognizes macrosialin, the murine homologue of the cluster of differentiation CD68, which is an endosomal marker in macrophages and dendritic cells (Rabinowitz and Gordon, 1991). CD68 expression is a useful indicator of endocytic activity, and the data suggest that BgPMs are less endocytically active than TPMs. Therefore, BgPMs may be useful in studies examining the entry and replication of facultative intracellular pathogens (e.g. *Mycobacterium tuberculosis*, *Mycobacterium leprae* and *Leishmania donovani*) within endosomal compartments. *In vivo*, elicited BgPMs, largely unstimulated by lymphokines, are permissive

Table 1. Phenotype of resident, elicited and activated murine peritoneal macrophages

	Resident	Thioglycollate	Biogel	BCG
Approx. total peritoneal cell yield per mouse ($\times10^6$)	7	21	17	10
Macrophages (%)	40	86	59	62
Adherence on TCP at 24 h	+	+	+/−	+
F4/80 expression	++	+	+	+
Mannose receptor	++	++	++	+
CD68 expression	+	++	+	+
MHC class II	−	+	+	+++
Induced respiratory burst	−	+	+	+
Constitutive NO production	−	−	−	+

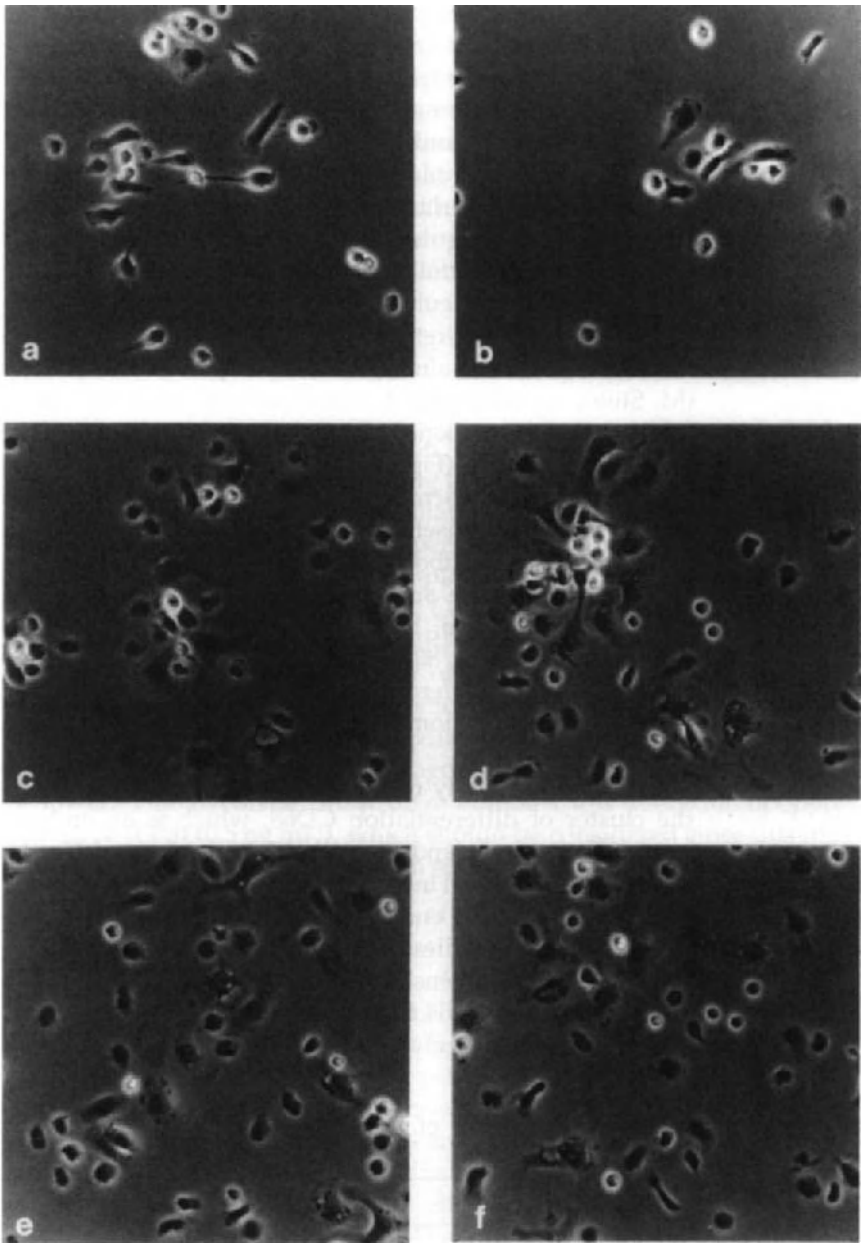

Figure 1. The effect of culture medium on BgPMs cultured on glass coverslips. Cells were harvested as described and allowed to adhere for 30 min in RPMI + 10% serum (R10) (a, b), in RPMI alone (c, d), or in Optimem (e, f). Cells were fixed in 2% paraformaldehyde and photographed under phase contrast . (a, c, e) Wild-type cells; (b, d, f) cells from mice lacking the class A scavenger receptor (SRKO). BgPMs spread more rapidly on this surface in media lacking serum (Optimem or RPMI). There is no discernable difference in the spreading morphology of wild-type cells and cells that lack macrophage scavenger receptor class A (SR-A). Original magnification ×400.

host cells for the above-mentioned pathogens (Gordon, 1986). Therefore BgPMs, or other foreign body elicited macrophages that resemble cells recruited early to a focus of infection, may be appropriate populations for studies examining the regulation of macrophage microbicidal activity. In addition, these cells respond well to cytokines *in vitro*, e.g. interleukin-4 (IL-4) and γ-IFN (Stein *et al.*, 1992). If sepharose, polystyrene or smaller polyacrylamide beads are used, then a higher percentage of PMNs will be recruited.

BCG recruited cells

BCG organisms (e.g. Pasteur strain) provide a suitable stimulus to recruit immunologically activated macrophages to the peritoneal cavity (Ezekowitz *et al.*, 1981). BCG stocks are stored at –70°C and thawed immediately prior to use. The BCG organisms are resuspended in PBS and sonicated before use. Mice are inoculated with approximately 10^7 colony-forming units (cfu) in 0.2 ml PBS by intraperitoneal injection. Peritoneal macrophages are harvested by lavage, as described above for RPMs, at 4–6 days post-injection. Percoll gradients can be used at this stage in order to enrich the population for macrophages (Pertoft and Laurent, 1977).

These macrophages become activated *in vivo* under the influence of T-cell products, such as IFN-γ, and express high levels of cell-surface major histocompatibility complex (MHC) class II. In addition, they produce nitric oxide in serum-containing media in the absence of further stimulation, in contrast to the other elicited macrophage populations described above (Fig. 2). These cells are also useful for investigating the activated macrophage response to bacterial cell products such as LPS and lipoteichoic acid (LTA), which can be added to cells in culture.

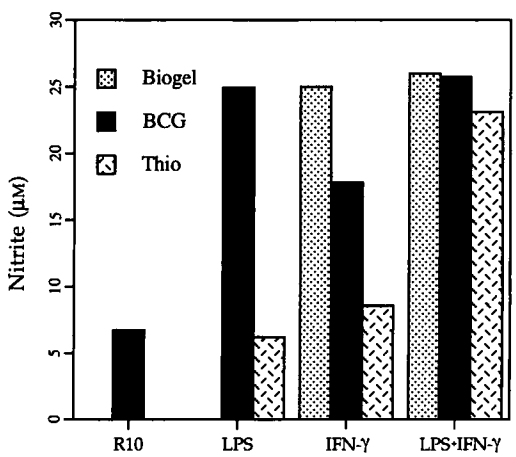

Figure 2. Nitric oxide production by thioglycollate (thio), Biogel and BCG recruited peritoneal cells *in vitro*. Cells were harvested as described in the text, plated at 2×10^5 cells per well and treated with medium (R10) alone, LPS (20 ng ml^{-1}) or IFN-γ (100 units ml^{-1}) overnight. Nitrite levels were measured using the Griess reaction, as described in the text. All values shown are L-*N*-monomethyl arginine inhibitable nitrite accumulation.

Corynebacterium parvum recruited cells

The use of inactivated *Corynebacterium parvum* (also known as *Propionibacterium acnes*) provides a convenient, alternative method of recruiting activated macrophages. The use of this organism to recruit macrophages to the liver of mice has recently resulted in the cloning of a new cytokine (IL-18) that induces production of IFN-γ by T cells (Okamura *et al.*, 1995). The use of *C. parvum* avoids the need for using viable pathogenic organisms. Inactivated *C. parvum* whole cells can be purchased from RIBI Immunochemical Research Inc. The *C. parvum* is washed in non-pyrogenic saline twice, resuspended in PBS, and sonicated before use. Mice are inoculated with 500 μg in 0.2 ml PBS by intraperitoneal injection. Peritoneal macrophages are harvested by lavage, as described above, at 4–6 days post-injection.

Tissue Macrophages

Resident macrophages can be isolated from a range of different tissues. In addition, activated cells can be isolated from infected or inflamed organs using the enzymatic methods described below.

Spleen and thymus

Macrophages can be isolated from the spleen and thymus as follows. The organs are removed from the mice and stored in PBS on ice until used. They are then digested in 0.05% collagenase (Boehringer Mannheim) and 0.002% DNase (Boehringer Mannheim) in RPMI 1640 at 37°C for 30 min, in the absence of serum. The organ fragments are then mechanically disrupted by vigorous pipetting and the suspension filtered through a cell strainer (Falcon, Becton Dickinson). The macrophages can then be purified through an adhesion step as described above for BgPMs.

Note that in order to maintain macrophage cell integrity, it is important that sufficient digestion has taken place before mechanical disruption is used. It is worth noting that the macrophages in the spleen represent a heterogenous population, and different approaches may be used to isolate macrophage subpopulations.

Bone marrow

Both resident bone marrow macrophages (RBMMs) and bone marrow-derived macrophages (BMDMs) can be obtained from this tissue as follows.

Resident bone marrow macrophages

Mice are killed and the abdomen and hind legs sterilized with 70% ethanol in water. Following a transverse cut through the skin of the abdomen, the skin is dissected away from the abdomen and hind legs.

Using fine scissors the muscles attaching the hind limb to the pelvis and those attaching the femur to the tibia are removed. Only when the femur is well exposed should the tibia be cut through just below the knee joint using strong scissors, and the femur freed from the mouse by cutting through the pelvic bone close to the hip joint. The femur can then be stored in RPMI 1640 on ice until all femurs are collected. Place the bones in a Petri dish of 70% ethanol for 1 min to maintain sterility, before washing twice with PBS.

Next, each femur should be held firmly with forceps and, in a single motion, the expanded ends (epiphyses) cut off using strong scissors. Using a 5-ml syringe attached to a 25-gauge needle, the bone marrow should be flushed out by forcing an RPMI solution containing 0.05% collagenase and 0.001% DNase down the central cavity. Repeat the process from the other end of the bone. The marrow from two femurs is suspended in 10 ml of the same enzyme solution and digested at 37°C, with shaking, for 1 h. Digestion is then stopped by adding fetal calf serum (FCS) to a final concentration of 1% v/v. At this stage the marrow plug fragments should no longer be visible, and a homogenous suspension is obtained.

Clusters of cells can be enriched by gravity sedimentation in RPMI containing 30% FCS or by use of a Ficoll-Hypaque cushion (Pharmacia) (Crocker and Gordon, 1985, 1986). Purified cell clusters can then be washed twice in RPMI by centrifugation at 100*g* for 10 min, suspended in R10 and added to glass coverslips in tissue culture plates. After 3 h of incubation at 37°C , non-adherent cells can be washed off with PBS. This results in a population of adherent cells with the characteristic morphology of RBMMs, contaminated with a varying population of monocytes and neutrophils.

Bone marrow derived macrophages

To obtain BMDMs, femurs are flushed out with RPMI, with no enzymes added. Filter the resulting cell suspension through a 70-μm cell filtration unit (Falcon) and spin at 1200 rpm for 5 min.

Make Tris buffered ammonium chloride (TBAC) lysis buffer by mixing 0.15 M ammonium chloride and 0.17 M Tris in the ratio 9 : 1 and adjust the pH to 7.2. Filter sterilize before use.

Lyse any red cells by resuspending in lysis buffer for 5 min at room temperature and then washing three times in RPMI 1640.

Resuspend the cells at 5×10^5 cells ml^{-1} in R10 containing recombinant macrophage colony-stimulating factor (M-CSF). Alternatively, L cell conditioned media can be used as a source of M-CSF (Hume and Gordon, 1983). Incubate cells for 3 days in a bacterial plastic culture plate, then wash the plate to remove non-adherent cells and replace with fresh medium. At days 7–8, adherent cells can be removed by incubation with 5 mM ethylenediamine tetraacetic acid (EDTA) in PBS. These cells represent mature, proliferating macrophages, which can be used in assays. For example, these cells are capable of mediating phagocytosis of a wide range of particles and represent a highly responsive target population to

assay incorporation of [^{3}H]thymidine after restimulation by CSF-1 (Hume and Gordon, 1983).

Fetal liver

The fetal liver contains the richest source of macrophages in the developing mouse. F4/80$^+$ membrane processes of these cells interact extensively with developing haemopoietic cells, forming cell clusters *in vivo*. To investigate the interactions between erythroid cells and stromal macrophages, isolation of haemopoietic cell clusters is recommended (Morris *et al.*, 1988).

Peripheral Blood Mononuclear Cells

To obtain peripheral blood mononuclear cells (PBMCs), mice are killed and bled by cardiac puncture into a heparinized syringe with a 25-gauge needle. Blood is diluted by adding an equal volume of 0.9% saline, and layering over a Nycoprep 1.077 Animal cushion (Nycomed Pharma AS). Cells are centrifuged at 1900 rpm (no brake) for 15 min. Mononuclear cells are collected from the interface between the plasma and the Nycoprep cushion. These cells are resuspended in TBAC lysis buffer to remove contaminating red cells and then resuspended in RPMI/10% FCS (R10) before use.

Use of Cell Lines

A number of different cell lines can be used in assays for investigating the functions of professional phagocytes (Ralph, 1986). Murine macrophage-like cell lines include the widely available RAW 264 (Raschke *et al.*, 1978), J774 (Ralph *et al.*, 1975) and P388D1 (Koren *et al.*, 1975). These cells can be cultured in RPMI 1640 on either bacteriologic plastic (BP) or tissue culture plastic (TCP) surfaces.

◆◆◆◆◆◆ CULTURE OF MACROPHAGES

Following isolation of primary macrophages as outlined above, it is important to maintain the cells in culture under appropriate conditions. These conditions will vary according to the functional tests required. For example, the substratum on which the cells are growing may be important if adhesion studies are planned.

Substratum

Macrophages can be cultured in suspension using tissue culture vessels with a Teflon-coated surface (Nalgene). In contrast, macrophages adhere firmly to TCP (Falcon, Becton Dickinson) and BP (ICN Pharmaceuticals

Inc.). On BP, in the presence of serum, they can be readily harvested by incubation with PBS plus 5 mM EDTA for 30 min, prior to collection by pipetting. On TCP, harvesting requires the addition of 5 mM EDTA and 4 mg ml^{-1} lidocaine hydrochloride (Sigma) for 30 min (Rabinovitch and de Stefano, 1976). Trypsin is ineffective at removing macrophages from this surface. Cells treated in this manner should be centrifuged and washed thoroughly with fresh medium before use.

Media and Sera

Macrophages are routinely cultured in a wide range of media in our laboratory, including RPMI 1640, modified Eagle's medium (MEM) and Dulbecco's modified Eagle's medium (DMEM) (all available from Gibco). These media are supplemented with 2 mM glutamine, 50 IU ml^{-1} penicillin and 50 μg ml^{-1} streptomycin (Gibco). RPMI 1640 is also supplemented with 10 mM *N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulfonic acid] (HEPES) (pH 7.3). FCS (Sigma) is heat inactivated at 56°C for 30 min, filter sterilized (0.22-μm pore size) prior to use and added to the media at 10% by volume.

Primary macrophages can be cultured for a variable time under serum-free conditions. Specifically, in endocytic assays the use of Optimem (Gibco), which is a proprietary serum-free medium, has proved useful. However, it should be borne in mind that macrophages adhere to substrata under serum-free conditions by way of adhesion molecules that have not been properly identified. This can result in practical problems in harvesting the cells from substrata, prior to their use in assays.

◆◆◆◆◆◆ MEASURING MACROPHAGE FUNCTION

Adhesion Phenotype

One of the key functions of a professional phagocyte is its ability to adhere both to other cells and to the extracellular matrix (ECM). In order to explore the cell surface molecules involved in this interaction, *in vitro* assays of adhesion of macrophages to artificial substrata (e.g. TCP), have been developed. These have provided a useful strategy for purifying macrophages from a mixed cell population (see previous section) and also for isolating reagents that interfere with this adhesion. For example, murine macrophages adhere to BP in the presence of serum in a divalent-cation-dependent fashion. Used as a screening strategy to develop novel monoclonal mAbs, the ability of a hybridoma supernatant to inhibit this adhesion produced the 5C6 mAb (Rosen and Gordon, 1987). This antibody recognizes the type 3 complement receptor (CR3), a leukocyte integrin. Subsequent studies have shown that this molecule has an *in vivo* role in adhesion, since 5C6 is able to block adhesion of macrophages to inflamed endothelium and recruitment to immunologically non-specific stimuli (Rosen and Law, 1989; Rosen *et al.*, 1989; Rosen, 1990).

In order to use this strategy to identify further molecules involved in adhesion, the investigator can vary (a) the phenotype of the macrophages added, (b) the presence or absence of chelators or other chemicals, or (c) the character of the substratum. For example, macrophages adhere to TCP in the absence of divalent cations. The use of an adhesion assay, as outlined below, allowed the identification of a further molecule involved in adhesion, the class A scavenger receptor (SR-A) (Fraser *et al.*, 1993). Prior to these assays this molecule had no known adhesive function.

Method

Macrophages are plated at 3×10^5 cells per well of a 96-well plate in the presence of various mAbs and chelators. Plates are incubated at 4°C for 30 min, then at 37°C for 90 min before washing to remove non-adherent cells. Adherent cells are fixed in methanol, and stained with 40% giemsa for 1 h. The level of adhesion is quantified by solubilizing the dye in methanol and reading the optical density at 450 nm (Fig. 3).

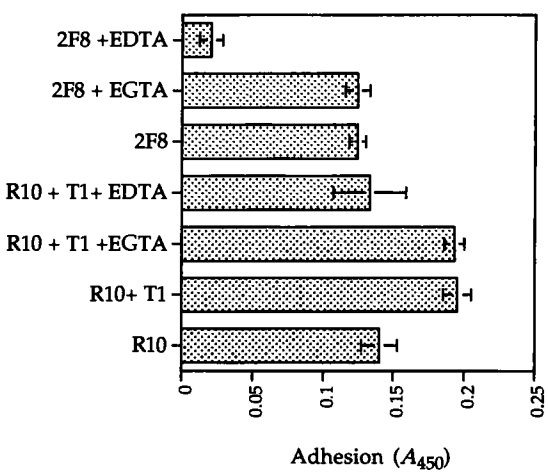

Figure 3. Adhesion phenotype of Biogel-elicited peritoneal macrophages. Adhesion of Biogel macrophages to fetal bovine serum (FBS) coated tissue culture plastic (TCP). Cells were plated at 3×10^5 macrophages per well of a 96-well plate in the presence of various mAbs and/or chelators (EDTA and ethylene glycol bis(b-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA)). Adhesion (mean ± SD) is represented as the absorbance at 450 nm (A_{450}), and is the result of quadruplicate wells. Significant adhesion occurs in the presence of an isotype matched antibody (T1) in the presence of EDTA. In contrast when 2F8 mAb is present, which blocks adhesion due to SR-A, then significant inhibition of adhesion occurs. Note that EDTA needs to be present to observe this activity, since other mechanisms of adhesion (e.g. integrin mediated) need to be inactivated.

Antigen Expression

Changes in levels of expression of either cell surface or intracellular antigens can provide useful information regarding activation status or endocytic activity of macrophages. A highly sensitive method of analysis

of individual cells is provided by immunostaining and flow cytometry. Immunohistochemistry is described elsewhere in this volume, and can be applied with success to defining resident and recruited macrophage populations *in vivo*.

Flow cytometry method

Elicited peritoneal cells are harvested, resuspended in R10 and plated onto TCP dishes. After 5 h, non-adherent cells are removed by repeated washing with PBS. Lidocaine (4 mg ml^{-1}) and EDTA (5 mM) in PBS is used to lift the macrophages from the plate. The cells are fixed on ice for 40 min using 4% paraformaldehyde/250 mM HEPES in PBS. If staining of an intracellular antigen is required, then the cells are permeabilized for 30 min at 4°C using saponin (0.1%) in PBS. Cells are resuspended in fluorescence-activated cell sorting (FACS) buffer (0.1% saponin, 0.1% bovine serum albumin (BSA), 1% normal mouse serum (NMS), 10 mM sodium azide) and incubated with primary antibodies (e.g. 10 μg ml^{-1} purified mAb) for 1 h, washed three times and incubated for 1 h with fluorescein isothiocyanate (FITC) conjugated mouse anti-rat second Ab (Jackson Immunoresearch) at 1:500 dilution. The cells are washed and analysed on a FACScan (Becton Dickinson) using Cellquest software.

Immunocytochemistry

Adherent cells are plated on 11-mm diameter acid-washed glass coverslips in 24-well plates, and allowed to adhere at 37°C in R10. After washing with PBS, cells are fixed for 10 min at 4°C in PBS containing either 0.125% glutaraldehyde or 2% paraformaldehyde (BDH, UK). Endogenous peroxidase activity can be blocked by incubation of sections with 10^{-2} glucose, 10^{-3} sodium azide, 40 U glucose oxidase in 100 ml 0.1 M phosphate buffer for 15 min at 37°C. To reveal intracellular antigens, cells can be permeabilized at this stage by incubation for 10 min at 4°C with 0.1% Triton-X 100 (BDH, UK).

Primary antibody (approximately 10 μg ml^{-1} purified, or neat tissue culture supernatant) is incubated with the cells for 1 h at room temperature in the presence of 5% normal goat serum. Antibody binding is detected with the appropriate biotinylated second antibody by the avidin–biotin peroxidase method (Hsu *et al.*, 1981), using reagents from Vector laboratories, UK, with diaminobenzidine as the chromagen. Mayer's haematoxylin can be used for counterstaining. Stained cells are dehydrated and mounted in DePeX mountant (BDH, UK).

Troubleshooting

One of the main challenges of immunostaining of monocytes or macrophages is to reduce the non-specific background staining (Gordon *et al.*, 1986). Often FcR (the receptor for the Fc portion of immunoglobulin (Ig)) or complement receptors are responsible for non-specific binding of

the primary or secondary antibodies used in the above methods. This binding can be reduced by (a) the use of fixed cells, and (b) the use of blocking serum appropriate to the antibodies used. In addition, great care should be taken to titrate the concentration of all antibodies used to ensure that staining is specific. A range of useful markers and their corresponding antigens is provided in Table 2. The stability of each antigen will vary according to fixation, and different fixation techniques should be tried to optimize results for each one. Incubation with FITC antibody should be in the dark. Include control cells that are incubated with either the first or second antibody alone. The latter is important in order to control for autofluorescence, which can be a problem with fixed cells. During analysis of a peritoneal cell population, investigate the population with high forward scatter. Macrophages tend to be larger than lymphocytes or neutrophils.

Endocytosis

Assays of endocytic activity can be used to examine the role played by specific cell surface receptors in the uptake of ligands from the culture supernatant (Gordon *et al.*, 1986). For example, the macrophage mannose receptor (MMR) is a cell surface receptor that binds mannosylated or fucosylated proteins. MMR activity is down-regulated on exposure to IFN-γ and upregulated by IL-4 (Stein *et al.*, 1992). Robust assays for binding, uptake and degradation of MMR ligands have been developed and are reported elsewhere (Ezekowitz and Gordon, 1982; Ezekowitz *et al.*, 1990). Selection of the appropriate ligand will depend on the specific receptor of interest. The example given below is the use of a fluorescently labelled modified lipoprotein (DiIAcLDL) for measuring uptake through a class of receptors known as scavenger receptors. These bind a wide range of polyanionic ligands, including modified lipoproteins and microbial components (e.g. LPS and LTA) (Dunne *et al.*, 1994). These receptors may play a role in atherogenesis and host defence (Krieger, 1992; Suzuki *et al.*, 1997).

Endocytic uptake of DiIAcLDL

Plate method

Elicited peritoneal cells are harvested and resuspended in RPMI plus 10% FCS (R10). Cells are plated at 3×10^5 macrophages per well of a 96-well plate and cultured overnight. Non-adherent cells are removed by washing with PBS. Medium (R10) is added containing either blocking or isotype matched antibodies, or polynucleotides that block uptake (e.g. Poly G), and the plate placed on ice for 30 min. Acetylated low density lipoprotein (AcLDL) labelled with 1,1'-dioctadecyl-1-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) (DiIAcLDL) (Biogenesis) (Pitas *et al.*, 1981) is added to each well at 5–10 μg ml^{-1} and the plate incubated at 37°C for 2–5 h. The cells are washed thoroughly and the dye solubilized

Table 2. Membrane antigens, and the corresponding antibodies, which can be used to define macrophage distribution and heterogeneity in murine tissues

Antigen	M_r ($\times 10^3$)	Antibody	Isotype	Tissue distribution	References
F4/80	150	F4/80	Rat IgG2b	Mature Macrophages	Austyn and Gordon (1981), McKnight *et al.* (1996)
CR3	150/90	5C6	Rat IgG2b	PMNs, macrophages and NK cells	Rosen and Gordon (1987)
7/4	40	7/4	Rat IgG2a	PMNs and activated macrophages	Hirsch and Gordon (1983)
Sialoadhesin	185	SER-4 3D6	Rat IgG2a	Stromal macrophages	Crocker and Gordon (1989), Crocker *et al.* (1991)
Macrosialin (murine CD68)	85–90	FA.11	Rat IgG2a	Macrophage and dendritic cell endosome membrane	Rabinowitz and Gordon (1991)
Class II (IA/IE)	α chain 35 β chain 30	TIB 120	Rat IgG2b	Up-regulated on activated macrophages	Bhattacharya *et al.* (1981)
Scavenger receptor type A	220–240	2F8	Rat IgG2b	Mature macrophages and hepatic endothelium	Fraser *et al.* (1993), Hughes *et al.* (1995)

with butan-1-ol. Fluorescence is read on a Fluoroskan II plate reader. Uptake of DiIAcLDL is expressed as units of fluorescence (mean ± SD) of four replicate wells.

Uptake of AcLDL by adherent cells assayed by microscopy

Cells to be tested are plated on glass coverslips in R10. After overnight incubation at 37°C, cells are washed and placed in the same medium containing DiIAcLDL at 5–10 μg ml^{-1}. After 3 h incubation at 37°C, cells are washed three times in PBS, mounted on microscope slides and viewed under epifluorescence microscopy using standard rhodamine excitation and emission filters.

Quantitation of AcLDL uptake by flow cytometry

Uptake of DiIAcLDL can be measured as described by Geng and Hansson (1992). Cells are plated in 24-well TCP plates at 10^6 cells/well in R10. After overnight incubation at 37°C, cells are washed and incubated in the same medium with 5 μg ml^{-1} DiIAcLDL, plus either 10 μg ml^{-1} mAb, 400 μg ml^{-1} unlabelled AcLDL (Biogenesis) or 200 μg ml^{-1} of scavenger receptor inhibitor (e.g. Poly G). After 3 h incubation at 37°C, cells are washed four times in PBA, and three times in PBS. Harvest the cells by pipetting, after incubation in PBS plus 5 mM EDTA and 4 mg ml^{-1} lidocaine. Cells are fixed in 2% (w/v) paraformaldehyde in PBS, and subjected to flow cytometry. Emitted fluorescence is collected at 563–607 nm (FL2 photomultiplier), and specific fluorescent intensity can be calculated by subtracting autofluorescent intensity from fluorescent intensity of DiIAcLDL labelled cells using geometric means derived from data analysis by Cellquest software.

Phagocytosis

Phagocytosis can be measured *in vitro* and this allows the cell populations and conditions to be well defined (Leijh *et al.*, 1986). By way of example different approaches to measuring the uptake of the yeast *Candida* are discussed here. Those seeking methods of assaying bacterial uptake are referred to previously published material (Langermans *et al.*, 1994).

Uptake of *Candida*

Candida can be phagocytosed in an opsonin independent or dependent process, depending on the presence of serum. An accurate assay has been described by Marodi, and this is outlined below (Marodi *et al.*, 1991).

Incubate 500 μl cell suspension containing 10^7 macrophages ml^{-1} with an equal volume of a suspension of 10^7 *Candida* ml^{-1} in Teflon-coated tubes in the presence or absence of serum at 37°C under rotation (4 rpm). At various time points, 50-μl aliquots of the mixture can be removed and added to 2 ml ice-cold PBS containing 50% FCS. Cytospin preparations

are made by spinning 100 µl diluted cell suspension onto each glass slide at 800 rpm for 5 min.

Alternatively, this assay can be carried out using adherent macrophages on coverslips. In this case, *Candida* are added and phagocytic uptake stopped at various time points by adding 2% paraformaldehyde in PBS.

After air drying the slides are stained with Diff-Quick stain (BDH). The percentage of ingested yeast is determined by viewing 200 high power fields and counting all extra and intracellular *Candida*. Yeast within detectable phagocytic vacuoles is considered intracellular. Alternatively, yeast conjugated with FITC (Sigma) can be used. This allows fluorescence of extracellular *Candida* to be extinguished by addition of 0.5 mg ml^{-1} Crystal Violet. This technique has helped differentiate between attachment of the yeast to the cell surface and internalization of the yeast by the cells. The ability to distinguish between these two processes is of prime importance to the characterization of the infection process, since the ingestion phase has been regarded as the rate limiting step in the killing of some micro-organisms (Hed, 1977). For practical purposes, binding of *Candida* to macrophages can be defined as a close association between at least 25% of the circumference of the yeast particle and the macrophage plasma membrane.

More rapid assays using a spectrofluorometric approach have been described (Ragsdale and Grasso, 1989). These have also been adapted for use when measuring bacterial phagocytosis (Plasman and Vray, 1994). They are convenient, but do not provide the level of accuracy or cellular information provided by the microscopic method outlined above.

Secreted Products

An important functional characteristic of macrophages is their conversion, under appropriate stimulation, from the resting to the activated state. Activated macrophages have increased numbers of lysosomal granules, more mitochondria and a greater capacity to phagocytose opsonized particles. In addition, the activated cell produces higher levels of certain cytokines (e.g. tumour necrosis factor α (TNFα)) and has an increased capacity to generate superoxide anions. Assays for measuring levels of superoxide, nitric oxide and cytokines *in vitro* are described below.

Superoxide

Plate macrophages at 1×10^5 to 5×10^5 per well in R10 in a 24-well tissue culture plate. Suitable negative controls include a cell-free blank and wells containing superoxide dismutase at 30 µg ml^{-1}. Positive controls should include wells containing elicited macrophages stimulated with phorbol 12-myristate 13-acetate (PMA) (Sigma) at 10–100 ng ml^{-1}, or zymosan (Sigma) at 100 µg ml^{-1}. Wash adherent cells once with PBS and incubate with 0.45 ml reaction mixture for 5 min at 37°C. Then add 0.05 ml Hanks buffered saline solution (HBSS) including the stimulant.

The reaction mixture comprises:

- HBSS
- 80 μM ferricytochrome C (Sigma, type IV)
- 2 mM sodium azide (prevents re-oxidation of cytochrome C)
- 10 mM sodium phosphate buffer, pH 7.4.

After 60 min incubation at 37°C, remove 100 μl supernatant from each well and read the absorbance at 550 nm against a reaction mix cell blank. This assay provides an easy and convenient method for estimating microbicidal and cytocidal potential. Other assays of respiratory burst activity include hydrogen peroxide release (Root *et al.*, 1975) and chemiluminescence (Trush *et al.*, 1978). The release of superoxide from murine macrophages is tightly regulated, and therefore freshly isolated cells produce neglible levels of superoxide in the absence of further stimulation. This results in a low baseline to an assay which is robust and reproducible.

Nitric oxide

Nitric oxide (NO) is a highly reactive molecule that mediates cytotoxic effects on micro-organisms and tumour cells (Saito and Nakano, 1996). Recently, NO has become an important player in innate immunity as a candidate mediator of macrophage cytotoxicity against intracellular pathogens (Nathan, 1995; Nathan and Hibbs, 1991). For example, the induction of NO production following BCG infection has been known for some time (Stuehr and Marletta, 1987).

Because NO is rapidly converted to nitrite in the presence of oxygen, the secretory activity of cells can be estimated by determining nitrite concentrations by the colorimetric Griess reaction outlined below.

1. Plate cells in a 24-well plate at 1×10^5 to 5×10^5 cells per well in 300–500 μl per well of R10, or in 96-well plates at 10^5 cells per well.
2. To remove non-adherent cells, wash with PBS after 3 h adherence at 37°C and add fresh R10.
3. Add stimuli, e.g. LPS at 20 ng ml^{-1} (Sigma) with or without IFN-γ at 50–100 units ml^{-1} (Serotec).
4. For each stimulus set up a negative control with 250 μg ml^{-1} *N*-monomethyl arginine (NMMA) (Sigma) the same time as the stimulus.
5. Incubate the plate overnight at 37°C.
6. Remove 100 μl supernatant to a 96-well plate. Set up sodium nitrite doubling dilutions, starting with 1 mM nitrite to calibrate the assay. Add 100 μl of fresh Griess mix to each well, prepared by mixing 1:1 the following solutions:

- 1% w/v naphthylethylenediamine diHCl (Sigma) in distilled water
- 1% w/v sulfanilamide in 5% v/v phosphoric acid (Sigma).

Both solutions can be stored at 4°C for up to 2 months.

7. After 10 min at room temperature, remove cellular debris by spinning the plate at 600 rpm for 10 min. Then decant the supernatant into a new plate. If bubbles are present, spin the plate at 3000 rpm for 1 min.
8. Read the absorbance at 550 nm. Express results as NMMA inhibitable accumulation of nitrite per 10^6 cells.

Cytokines

Murine macrophages secrete a wide range of cytokines *in vivo* and *in vitro*. It is possible to assay cytokine concentrations from both serum and from culture supernatant. Cytokines may be assayed by use of either bioassay or enzyme-linked immunoabsorbent assay (ELISA). By way of example, protocols for the measurement of TNFα are given below.

Bioassay. This is a useful assay which enables measurement of bioactive TNFα. L929 cells represent the targets and are susceptible to lysis when TNFα is present above a certain concentration.

1. *L929 cell culture.* Grow L929 cells in Eagle's minimum essential medium (EMEM) or DMEM containing 24 mM HEPES, 5% FCS at pH 7.4. It is important to split cultures (1:10) frequently from a non-confluent status and change the medium every 3–4 days. Use PBS to wash the cultures and incubate with trypsin (0.01%) (Gibco, BRL)/5 mM EDTA in PBS to detach the cells.
2. *L929 cytotoxic assay.*

- Assay medium: Cell culture medium supplemented with penicillin 50 IU ml^{-1}/streptomycin 50 μg ml^{-1}.
- Day 1: Detach cells from a semiconfluent status and resuspend in assay medium at $4 \times 10^5 ml^{-1}$. Dispense 4×10^4 cells in 100 μl medium in all wells except row 1, A–D (blanking wells). Incubate for 18–20 h at 37°C in 5% CO_2.
- Day 2: Aspirate and discard spent medium. Replace it in all wells with 100 μl assay medium supplemented with 1 μg ml^{-1} actinomycin D. Add in wells A2–A11 50 μl of the samples to be tested and 50 μl of medium containing 2 μg ml^{-1} actinomycin D. Add 50 μl of recombinant TNFα 10 ng ml^{-1} (Serotec) to well A12. Double-dilute down the plate with an eight-channel pipette from row A to row H (seven dilutions: ¼, ⅛, 1/16, etc.), discard the last 100 μl. Incubate for 18–20 h.
- Day 3: Discard the medium, replace with at least 40 μl Crystal Violet solution (0.5% in 20% methanol). After 2 min, wash vigorously with tap water. Dry and read absorbance in ELISA plate reader at 540 nm. Select the A1 well as a blank.
- Data processing: The average of column 1 will give absorbance

relative to the half-maximum unit. Express sample concentration as the reciprocal of the dilution which gives an absorbance of the half-maximum unit. It is important to compare, for plate to plate comparison, the standard reading obtained using recombinant TNFα. The appearance of a typical assay is provided in Fig. 4. If there is no lysis of cells in the positive control wells, then a common cause is that the L929 cells were either confluent before transfer to the 96-well plate or had overgrown in the plate before samples were added.

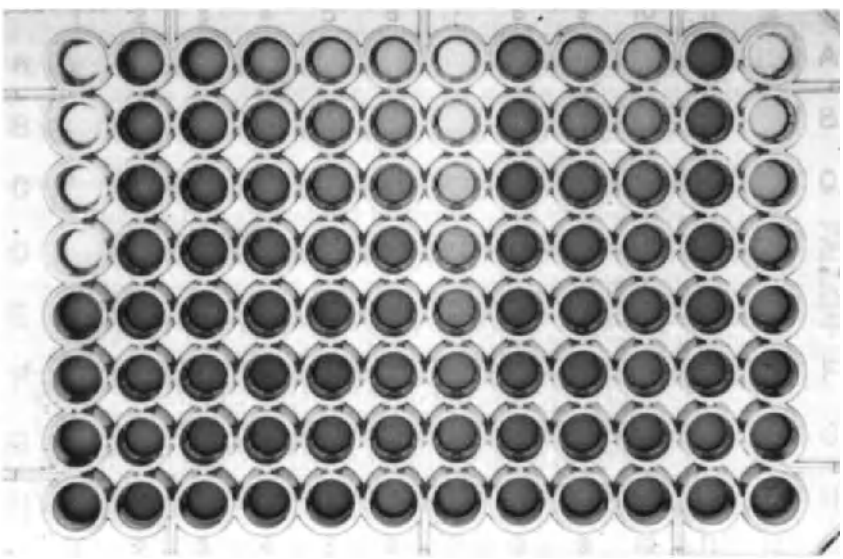

Figure 4. L929 Bioassay for TNFα. L929 cells were seeded onto a 96-well plate, as outlined in the text. Samples of culture supernatant were added to wells A2–A11 and a control sample of TNFα was added to well A12. Samples were double-diluted down wells. Remaining L929 cells have stained dark with Crystal Violet. From this assay it can be seen that column 7 received the culture supernatant containing the highest concentration of TNFα.

ELISA. The concentration of TNFα in serum or culture supernatant can be determined using a TNFα capture ELISA. Briefly, 50 µl TN3 19.12 mAb 4 µg ml^{-1} in carbonate buffer pH 9.6 is added to each well of a 96-well plate (Titertek, ICN) and left at 4°C overnight. Plates are washed twice with PBS/0.05% Tween 20 (Sigma) and twice with PBS alone. Then, samples of the sera or supernatant are added, along with a dilution series of recombinant murine TNFα (Serotec, Oxford, UK). Plates are incubated overnight at 4°C, washed as above, and 100 µl rabbit anti-murine TNFα (Serotec) (1 : 1000 in PBS) is added to each well and the plate incubated at room temperature for 90 min. Plates are washed as above, donkey anti-rabbit conjugated to horse radish peroxidase (HRP) (Chemikon) at 1 : 1000 in PBS/(0.05%) Tween/(0.1%) BSA is added and incubated at 20°C for 1 h.

Plates are washed, and 100 µl reaction mixure added as recommended by the supplier. The reaction is stopped by addition of 50 µl 3 M sulphuric acid and plates are read at 492 nm.

If all wells in the ELISA develop a positive reaction, including the negative controls, then inadequate washing of the plate after incubation periods is the likely cause. If no reaction occurs, even in the positive control wells, then a frequent cause of failure is omitting to add all constituents of the reaction mix.

◆◆◆◆◆◆ CONCLUSION

The assays described here will enable investigators to isolate various macrophage populations from the mouse and measure their functions in a range of assays. The choice of populations is an important part of the experimental design. Table 1 shows that the cellular phenotype can vary widely according to stimulus, even when cells are isolated from the same site. Cell surface receptors expressed on different macrophage populations can be recognized using a wide range of different cell surface markers (Table 2).

The mouse represents an important source of primary cells for use in studying the cellular response to micro-organisms. The development of transgenic and gene knockout mice has provided new tools for the study of the role of, for example, cell surface receptors or cytokines in the binding, uptake and killing of microbial pathogens. In addition, rapid progress is now being made in elucidating the molecular biology underlying many aspects of function (Gordon *et al.*, 1995). Further investigation using these new tools will hopefully enhance our understanding and characterization of the host–pathogen relationship.

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3 *In Vivo* Analysis

3.1 The Immune Response in Mice Challenged with Bacterial Infections

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◆◆

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I hope we never lose sight that all this was started by a mouse.
Walt Disney

◆◆◆◆◆◆ INTRODUCTION

Human interest in immunology dates back to ancient times when man first realized the phenomenon of acquired resistance against infections. Taking into consideration also the fact that severe infections are one of the major driving forces in the evolution of the immune system, since its beginnings the study of immunology has been closely related to studies of host defence mechanisms.

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Table 1. First descriptions of phenomena associated with host response to infectious agents

Experience		
Fever	Mesopotamia	3000 BC
Cardinal signs of inflammation	Celsus	25 AD
Variolation for smallpox	China	1000 AD
Controlled observation		
Cowpox vaccination	Jenner	1798 ('human experiment')
Experiment		
Animal experiment in physiology	Bernard	1865
Attenuated vaccines	Pasteur	1880
Protective antibodies	von Behring, Kitasato	1890
Delayed hypersensitivity in tuberculosis	Koch	1890
Anaphylaxis	Richet, Portier	1902
Arthus phenomenon	Arthus	1903
Passive transfer of cell-mediated immunity	Chase, Landsteiner	1945
Classification of immunopathological mechanisms	Gell, Coombs	1962

Table 2. Well-established murine models of bacterial infections and intoxications in humans

Non-replicating agents

Systemically acting bacterial toxins:
- Tetanus toxin
- Botulinus toxin
- Toxic shock syndrome toxin-1 (TSST-1), *Staphylococcus* enterotoxin B (SEB) (*S. aureus*)

Locally acting bacterial toxins:
- Enterotoxins (*Escherichia coli, Clostridium difficile*)

Replicating agents

Peritonitis:
- *Escherichia coli*
- *Bacteroides fragilis*
- *Enterococcus faecalis*

Enterocolitis:
- *Salmonella enteritidis**
- *Yersinia enterocolitica**

Table 2. *contd.*

Cystitis, pyelonephritis, cervicitis, pelvic inflammatory disease:
- *Chlamydia trachomatis* biovar mouse pneumonitis agent (MoPn)
- *Escherichia coli** (uropathogenic strains)
- *Proteus mirabilis**

Pneumonia:
- *Haemophilus influenzae* type B (bacteraemia, meningitis)
- *Klebsiella pneumoniae** (bacteraemia)
- *Pseudomonas aeruginosa** (bacteraemia)
- *Streptococcus pneumoniae** (bacteraemia, meningitis)
- *Coxiella burnetii*
- *Legionella pneumophila*
- *Mycobacterium tuberculosis*

Dermatitis, arthritis:
- *Staphylococcus aureus**
- *Pseudomonas aeruginosa*
- *Borrelia burgdorferi* (SCID mouse model)

Systemic infections
Extracellularly replicating agents:
- Gram positive
  - *Staphylococcus aureus* (endocarditis, arthritis)
- Gram negative
  - *Pseudomonas aeruginosa* (meningitis; immunocompromised (neutropenic) host)
  - *Haemophilus influenzae* type B (meningitis)

Intracellularly replicating agents:
- Gram positive
  - *Listeria monocytogenes** (meningitis/encephalitis)
  - *Rhodococcus equi* (pneumonia)
- Gram negative
  - *Bartonella bacilliformis*
  - *Brucella abortus*
  - *Francisella tularensis**
  - *Mycobacterium tuberculosis*
  - *Mycobacterium avium*
  - *Rickettsia akari, R. tsutsugamushi*
  - *Salmonella typhimurium*
  - *Yersinia enterocolitica**
  - *Yersinia pseudotuberculosis**

*Also described as spontaneous infections in mice.

Due to extensive *in vitro* studies, much is known about the cells and molecules of the human immune system that are accessible in blood. However, relatively little is known about the way in which these cells and molecules behave or are controlled *in situ*, especially in the mucosa-associated lymphoid tissue, the skin, parenchymal (non-lymphatic)

organs such as the liver and lung, or privileged sites such as the central nervous system, even though infections of concern in medicine are commonly those of the aforementioned organs. Important variations in local immunity may be demonstrated by comparing the host response to a particular agent in different organs as exemplified by the heightened susceptibility of the lung to *Mycobacterium tuberculosis*, the brain to *Cryptococcus neoformans*, or the placenta to *Brucella abortus*. On the other hand, studying the immune response in one organ system to various infectious agents will reveal typical patterns of response at this locality.

Although the era of modern immunology started with a controlled observation in humans (Jenner, Table 1), there are obvious reasons for using animals instead of humans to elucidate bacteria-induced mechanisms of protective immunity and inflammation (Table 2). The impact of animal models on the development of immunology can best be estimated by a historical overview, which demonstrates that most discoveries in the field of immunology have been made since the inauguration of experimental methods in animals (see Table 1).

The analysis of bacterial infections in animal models usually focuses on three topics:

- the mechanisms by which a bacterium induces the tissue lesions that result in the symptoms of disease (pathogenesis)
- the mechanisms of host defence against the invasive organism or its toxins
- the evaluation of antimicrobial or immunomodulating agents such as antibiotics, adjuvants, cytokines, vaccines and antibody preparations.

While there is no doubt about the usefulness of animal models in the field of immunology, the question is which species is best suited to be used as an experimental subject.

Murine Models of Medically Important Bacterial Infections

From an evolutionary point of view, it is obvious that animals that have been exposed to a comparable selective pressure, that have developed an immune system of the same level, that live in close contact with man, and that have the same body temperature, for example rodents, are better suited as models than are birds, reptiles or amphibious animals. In fact, murine models of disease have contributed much to our understanding of infectious diseases and immunology. Nevertheless, mouse models are often chosen just because of habit, their inexpensiveness, their small size (easier handling and housing) and the relative lack of public sympathy for them. This, in turn, has at least led to a self-enhancing effect, resulting in the availability of genetically defined inbred strains, transgenic and gene knockout (GKO) mice, and a plethora of mouse-specific reagents that can characterize the immune response (e.g. monoclonal antibodies (mAbs) and recombinant cytokines). Consequently, a tremendous amount of knowledge about the immune system of the mouse has been accumulated.

However, before mice may be used as a model of human immune responses to bacteria, the following questions have to be answered:

- Does the experimental infection of mice mimic natural disease in humans, i.e. is the host response comparable?
- Is the degree of resistance against the challenging pathogen comparable to that in man?

Bacteria that are much less virulent, are unable to infect via a natural route, have a different organ tropism, or lead to different symptoms or histopathological changes in mice than in man should not be used as stimuli in studies of pathogenesis and immune response.

Although there are a lot of similarities between the immune systems of man and mice, even at the molecular level (e.g. cross-activity of various cytokines, with the exception of interleukins-3, -4 and -12 (IL-3, IL-4, IL-12) as well as γ-interferon (IFN-γ), see Table 11), there are some significant differences that are of importance in the interpretation of results obtained in murine models:

- *Blood.* There is an inverse relationship between the number of circulating polymorphonuclear cells (PMNs) and lymphocytes in man and mice (see Table 6). It is astonishing, however, that the inflammatory response in various tissues is often quite similar. This is an important example of the fact that the analysis of blood may be misleading in terms of characterizing host responses.
- *Spleen.* The spleen of the normal adult mouse routinely functions as a haematopoietic organ. One or more nodules of accessory splenic tissue are often embedded in the adjacent pancreas, which is of importance in the interpretation of results obtained after splenectomy. The vascular arrangement in the spleen is somewhat different in man and mouse, the latter demonstrating a higher proportion of blood circulation through tissue spaces rather than through sinuses.
- *Liver.* As is the case in spleen, the liver of mice is capable of haematopoiesis during adulthood. This may lead to confusion of, for example, megacaryocytes or clusters of erythoblasts with giant cells or inflammatory infiltrations. In contrast to human liver, trabeculae in the murine liver consist of monolayers of hepatocytes that are not separated from blood by a basal membrane, which may result in different mechanisms of bacterial invasion.
- *Lung.* Bronchus-associated lymphoid tissue (BALT) is not present in normal mouse lung. There are no submucous glands in the lower respiratory tract of mice. There may, however, be sparsely distributed nodules of tightly packed lymphocytes within the lung parenchyma of normal mice, which should not be confused with inflammatory lesions.
- *Intestinal tract.* Likewise the gut-associated lymphoid tissue (GALT) differs in some respects from that of humans. The mouse lacks palatine and pharyngeal tonsils and has no vermiform appendix. Instead, the caecum of mice resembles the human appendix with regard to the lymphoid tissue. However, while the human intestine contains about 100 Peyer's patches within the ileum, each having 20–25 lymphoid follicles, the mouse intestine (throughout the whole small intestine) contains 8–12 Peyer's patches with 4–10 lymphoid follicles, depending on the mouse strain. The number of M

cells within the follicle-associated epithelium of Peyer's patches is also species and even strain specific, and this may have profound effects on the invasion of bacteria from the gut.

In addition to these differences at the organ level, there are some systemic variations in the immune systems of man and mice:

- The cellular distribution of major histocompatibility complex (MHC) products is similar in man and mouse, but not identical. MHC class I gene products are expressed on virtually all nucleated cells in both species and on murine, but not on human, erythrocytes.
- The total amount of immunoglobulins in serum is lower in mice (2.3–6.6 mg ml^{-1}) than in humans (9.5–25.3 mg ml^{-1}).
- In mice, serotonin is more important than histamine. Thus, anaphylactic shock may not be as violent in mice as in other species.

These examples demonstrate that detailed knowledge about the anatomical and physiological differences between humans and mice may be critical for the correct planning and interpretation of animal experiments. Despite these differences, however, there are well-established murine models of medically important bacterial infections (Tables 2 and 3). These have been shown to be valid models of the host response to replicating antigens, and have contributed much to our knowledge about the immune system (Mielke *et al.*, 1997). Here we focus on those methods that have a demonstrated value of providing important information about the immune system when mice are challenged with bacteria.

Table 3. Infectious agents behaving differently in man and mice

Disease	Human pathogen	Murine equivalent
Gonorrhoea	*N. gonorrhoeae*	No equivalent
Syphilis	*T. pallidum*	No equivalent
Typhoid fever	*S. typhi*	*S. typhimurium*
Enteritis	*S. typhimurium*	*S. enteritidis*
	Esch. coli (enteropathogenic)	No equivalent
	C. jejuni	Not pathogenic if administered orally
	Shigella spp.	Not pathogenic if administered orally
Pneumonia	*M. pneumoniae*	*M. pulmonis*
	C. pneumoniae	*C. trachomatis* biovar MoPn
Cholera	*V. cholerae*	No equivalent (suckling mice may be used)
Erysipel	*S. pyogenes*	No equivalent
Reactive arthritis	*Y. enterocolitica*	No equivalent
Lepra	*M. leprae*	*M. lepraemurium*
Leptospirosis	*Leptospira interrogans*	Carrier status
Peptic ulcer, cancer	*Helicobacter pylori*	*H. hepatis?*

◆◆◆◆◆◆ EXPERIMENTAL INFECTION AND IMMUNIZATION OF MICE

Immunological experiments most often start with the immunization of animals. In contrast to several other antigens, viable bacteria are usually strong immunogens so that, in most cases, no additional adjuvants are needed for immunization. However, using viable agents for immunization, several parameters including the source, propagation, preparation and storage of the bacteria, have to be carefully controlled to ensure reproducibility and comparability of the data obtained in different laboratories or in the same laboratory at different times.

Sources and Handling of Infectious Agents

Propagation, inactivation and storage of bacteria

Whenever working with a pathogen is being considered, the first thing to do is to look for guidelines about safety precautions and effective methods for inactivation of the agent. As this information may change due to legal ramifications, we strongly advise the reader to look for the newest information available on this topic. It should be emphasized, however, that in any case the following bacteria have to be handled under the most stringent safety conditions because of the high incidence of accidental infection: *B. abortus, Coxiella burnetii, Francisella tularensis, Legionella pneumophila* and *M. tuberculosis*. Ideally, the bacterial species used to study experimentally induced host responses is a species that causes disease in humans (see Table 2). However, some species that cause human disease do not cause illness in mice (see Table 3). A possible solution to this problem may be to use a closely related bacterial species that produces symptoms in animals that resemble human disease. Another strategy is to administer the bacteria intravenously or intraperitoneally, thereby bypassing mechanisms of mucosal invasion and mucosal host defence. For studies on pathogenesis, immunodeficient animals such as suckling or infant mice or animals immunocompromised by corticosteroids or on a genetic basis (e.g. severe combined immunodeficiency (SCID) mutation) may be used, although their limitations should be kept in mind and the results should not be overinterpreted.

The choice of which strain of a particular pathogen to use also poses problems, since different strains of one species may vary considerably in their ability to cause disease. In fact, differences in virulence between the various strains used in different laboratories often explain major discrepancies in the role of certain defence mechanisms. Therefore, immunological studies using infectious agents are of no value without information about the route of infection and the LD_{50} of the infective organism. Furthermore, pathogens may alter their phenotype considerably once they are moved from the human environment to artificial culture media. Therefore, it is advisable to preserve stock suspensions of the pathogen very soon after their primary isolation from humans. In any case, repeated passages *in vitro* must be avoided.

Sources of bacteria used in animal experiments are clinical isolates or bacteria obtained from culture collections such as American type culture collection (ATCC) (Rockville, MD) or its national equivalents. While clinical isolates often retain their virulence mechanisms, they are sometimes poorly defined and can give rise to results that cannot be reproduced in other laboratories working with another strain of the same species. On the other hand, bacteria obtained from culture collections may have lost their virulence due to serial subcultures in the absence of selective pressure. It may be that the only way out of this dilemma is to use and compare bacteria from both types of source.

While virulence in mice can often be increased and maintained by at least three initial cycles of *in vivo* passages, this method fails if virulence is encoded by genes on a plasmid that has been lost during *in vitro* culture. To obtain the initial culture, bacteria should be grown in rich media such as trypticase soy broth, brain–heart infusion or thioglycollate broth (Difco Laboratories, Detroit, MI) under optimal conditions. For *Borrelia* (BSK II medium + 12% rabbit serum, 32°C) and *Legionella* (BCYE agar, 37°C) special media are needed. *Chlamydia* (McCoy cell monolayer; overlay containing 1.5% agar) and *Rikettsia* have to be grown in tissue culture.

For experimental infections, bacteria from a log-phase culture are pelleted by centrifugation (3000*g*, 15 min) and washed once in sterile phosphate buffered saline (PBS). Aliquots of this stock suspension can be maintained at –70°C, or in liquid nitrogen in PBS or fresh broth. In some cases it may be necessary to include 15% glycerol. For immunization or challenge, aliquots of frozen stocks are thawed and diluted to the appropriate concentration with PBS. Differences in culture conditions may have profound effects on virulence, as the growth temperature and certain deficiencies in the culture medium (e.g. a low iron content) may result in reduced or enhanced expression of virulence factors. In addition, one should be aware that even the washing procedure may influence some characteristics of the bacteria, including virulence factors. For example, the capsule of bacteria may be sheared off by harsh centrifugation. In such cases, a dilution of more than 1 : 50 of the broth culture in PBS may be used *in vivo*.

In vivo passage of bacterial strains is a prerequisite for reproducible *in vivo* analysis of host responses to bacteria. For this purpose, a sublethal dose of bacteria should be administered intraperitoneally or intravascularly. After a certain time period, depending on the growth characteristics of the bacterium used (e.g. 1–4 days), the spleen is removed aseptically or a peritoneal lavage (5 ml sterile saline) is performed (see p. 344). The lavage fluid or organ homogenate is serially diluted and plated on agar, in addition to being inoculated into a series of broth cultures to obtain virulent bacteria. The bacteria should then be grown to log phase, again according to the requirements for the particular strain. Usually, a culture that shows only slight turbidity is chosen and the bacteria are collected by centrifugation, washed in PBS, frozen in aliquots and kept at –70°C or in liquid nitrogen. Prior to an experiment, the bacteria are thawed and grown to log phase again; alternatively, they may be injected directly. The viability and stability (in terms of virulence) of the frozen bacteria should

be determined prior to the experiment (e.g. by determining the number of colony forming units (cfu) just before and 24 h after freezing, as well as after a longer period of frozen storage) because the percentage of non-viable cells present after storage may vary dramatically and may significantly influence the type of immunization or outcome of challenge studies due to non-specific stimulation of defence mechanisms by non-viable bacteria.

The actual number of experimentally administered bacteria should be determined by plating serial 10-fold dilutions of the inoculum and counting the number of cfu after appropriate incubation. It should be borne in mind that virulence factors determining adhesion or invasiveness may be lost if passages are done bypassing mucosal surfaces by parenteral infection. On the other hand, since most invasive bacteria finally reach the bloodstream, the response to such strains may be analysed successfully if they are administered intravenously or intraperitoneally.

Since the course of disease is critically determined by the relationship between the replication rate of the bacterium *in vivo* and the onset of effective defence mechanisms, non-reproducible results may often simply be due to differences in the growth phase and replication rate of the bacteria used. Therefore, great care should be taken to ensure that bacteria used for *in vivo* experiments are used in the midlogarithmic phase in a standardized culture medium under standardized (optimal) culture conditions.

Counting bacteria

The number of viable bacteria in solutions can easily be determined by counting the number of colony forming units (cfu) on agar plates. However, to avoid falsely low bacterial numbers (e.g. due to clumping or adherence of bacteria), suspensions containing 0.1% Tergitol TMN 10 (Fluka, Buchs, Switzerland) and 0.1% bovine serum albumin (BSA) in PBS may be plated. A detergent in hypotonic PBS might also facilitate the release of intracellular bacteria from cells when numbers of bacteria in organ homogenates have to be determined. However, the effectiveness of this approach should be tested before use, as some bacterial species might be killed by the detergent.

Another method for determining the number of bacteria in a suspension just before infection is the nephelometric measurement of turbidity (e.g. at 660 nm in a Pharmacia LKB Ultraspec III). This has to be done in the midlogarithmic phase to ensure that turbidity is caused by viable organisms only. A retrospective confirmation by plating serial dilutions and determining the number of cfu is mandatory. Finally, bacteria may be counted in a Petroff–Hausser chamber (San Francisco Regional Distribution Center, Brisbane) by phase-contrast microscopy.

Preparation of non-replicating antigen from bacteria

Non-replicating preparations of bacteria may be used for the induction of an immune response, but more often they are used to elicit secondary

responses such as delayed type hypersensitivity (DTH) reactions and granuloma formation in primed mice.

Killed bacteria (particulate antigen) can be prepared by incubating PBS-washed bacteria of a midlogarithmic phase culture for 1 h at 63°C in a water bath. The number of cfu has to be determined before the preparation, and may be adjusted to $1 \times 10^{10} ml^{-1}$. The killed preparations may be stored in aliquots at –70°C. Alternatively, bacteria may be inactivated by treatment with 0.1% formalin or glutaraldehyde in PBS for 1 h at 37°C and subsequent washings in PBS. Another method is γ-irradiation of the bacteria with 25 Gy. Sterility must be confirmed by subculturing aliquots both in liquid media and on agar plates.

The preparation of soluble antigens can be achieved by ultrasonication of bacteria in PBS (1 g wet wt per 10 ml PBS) or by concentrating cell culture supernatants by ultrafiltration. Before use, soluble antigen preparations are filter sterilized using 0.2-μm filters. Progress in gene technology has enabled the generation of high amounts of pure proteins as recombinant antigens. These may be highly standardized tools for immunization and challenge. However, the lipopolysaccharide (LPS) content of such a preparation should be tested before use. Proteins should be purified according to the instructions of the manufacturer of the vector system used to produce the recombinant protein. Finally, the protein should, if possible, be solubilized in PBS or saline in order to avoid toxicity from compounds used during purification (e.g. urea). The amount of recombinant protein needed to generate humoral and/or cellular immune responses can vary significantly, and depends on the biochemical properties of the protein, on its immunogenicity and on the adjuvant used (see the chapter by Lövgren-Bengtsson, p. 471ff). For many proteins, 10 μg administered parenterally or intranasally is sufficient to generate an immune response. For orogastric immunization, a broad range of doses should be tested (10–100 μg per application).

Animals and Animal Facilities

Details about animal care and gene knockout as well as transgenic mice are given in the chapter by Mossmann *et al.*, page 109. Here we focus on those aspects that are particularly relevant to *in vivo* experiments involving viable bacteria.

Mice suited for immunological experiments can be purchased from the Jackson Laboratory (Bar Harbor, MN, USA), or other well-reputed distributors such as Harlan Sprague–Dawley. Inc. (Charles River Wiga, IN, USA), Harlan Olan Ltd, Bomhaltgard Breeding and Research Centre Ltd (Ry, Denmark), etc., that provide a health certificate including data about mouse pathogens such as mouse hepatitis or sendai virus. After transport, the mice should be allowed to rest for a few days in order to become acquainted with the new environment before the experiments are started. Stress due to shipping and handling may lead to a disruption of the normal circadian rhythm of corticosteroid secretion, which in turn may profoundly influence the immune response. The same is true for the

day/night rhythm, which should be standardized in all experiments. In addition, the water balance of the mice may be disturbed during travel, and the mice should be adapted to the food used during the whole experiment. In order to prevent social stress, no more than 10 mice should be housed in a 900-cm^2 cage.

For immunological studies, mice should at least have the standard of monitored animals, i.e. the mice should be housed in a low-security barrier system (change of laboratory coat, disinfection of hands, use of gloves and face mask, sterilization of cages) and should be demonstrated to be free of known pathogens by sequential monitoring (see the chapter by Mossmann *et al.*, page 109ff). Therefore, serum samples (see p. 343) should be drawn on a regular basis and analysed at least for the presence of antibodies against the most common pathogens such as mouse hepatitis and sendai virus.

Unknown latent or persistent infections are the major cause of non-reproducible experiments. Sources of infection that are often not realized are hybridoma and tumour cells used *in vivo*, and infections transmitted to animals from animal workers who have pets at home. The eradication of a viral pathogen such as mouse hepatitis virus may stop scientific work for at least half a year. Therefore, no foreign animals should be obtained

Figure 1. Microisolation housing units.

without being separated and controlled, and staff should be well educated.

Mice should be housed at 20–22°C (a temperature below 18°C or over 33°C will influence body temperature) at 60–70% humidity in air, and maintained on an alternating 12-h dark/light cycle. Mice may be fed heat-treated rodent chow and water *ad libitum*.

Experimentally infected mice should be housed in separate safety cabinets (Fig. 1). At the end of an experiment sacrificed animals should be collected in plastic bags and autoclaved (121°C, 20 min) before release from the laboratory.

Factors influencing susceptibility to infection

Susceptibility of mice to infections varies significantly with strain, age, body weight and sex. The immune response of very young mice differs from that of mature and of senescent mice. Adult levels of immune parameters are usually established after 9 weeks of age, and cell-mediated immunity decreases after the age of 24 months. Therefore, mature mice aged 10 weeks to 12 months should be used for immunological studies. Since susceptibility to infection may differ significantly in male and female mice, it is prudent to use only one gender throughout the entire study.

Susceptibility to oral infection may be influenced by the composition of the intestinal flora. Therefore, for each animal facility the microbial status 'specific pathogen free' (SPF) should be characterized in order to achieve reproducibility of experiments. For example, the presence of *Citrobacter freundii* in the intestinal flora is of particular interest in studies of gut-associated lymphoid tissue as this bacterium may account for transmissible murine colonic hyperplasia. Treatment of mice with streptomycin (Wadolkowski *et al.*, 1990; Lindgren *et al.*, 1993) is a well-known method of modifying the composition of the intestinal flora by reducing the facultative anaerobic bacteria that normally colonize the mouse intestine and thus to increase susceptibility for mucosal infection. For example, streptomycin-treated mice become susceptible to infection with *Esch. coli* F-18 and K-12. Susceptibility of mice to mucosal infections may also be modified by a vitamin A deficient diet (Wiedermann *et al.*, 1993). Vitamin A is an important regulator of growth, differentiation and proliferation of epithelial cells. Vitamin A deficiency reduces mucosal immune responses. Furthermore, it may lead to breakdown of mucosal integrity, and thus may increase susceptibility to mucosal infections. Quality of rodent chow is therefore an important feature.

Germ-free mice may be used for intestinal infection experiments. However, the behaviour of an infective agent might significantly differ in these mice, as bacteria from the normal flora often provide important colonization factors in the intestinal microenvironment. Furthermore, the development and differentiation of the gut-associated lymphoid tissue, including Peyer's patches and intraepithelial lymphocytes, depends on the presence of normal gut flora. Germ-free mice have only small Peyer's

patches, and the phenotypes of intraepithelial lymphocytes is changed (Umesaki *et al.*, 1993; Imakoa *et al.*, 1996). Likewise, the number of M cells within the follicle-associated epithelium (FAE) in reduced in these animals.

In some situations it might be helpful to initially immunosuppress animals by corticosteroids to get an infectious process started. This can be done by the subcutaneous injection of 125 mg kg^{-1} per animal once daily for 6 days.

Mouse inbred strains and spontaneous mutants

The discovery of spontaneous mouse mutants presenting with immunodeficiencies (e.g. nude or SCID mice) and the generation of inbred strains had a great impact on immunological research.

SCID mice lack the recombinase necessary for the rearrangement of T-cell receptor and immunoglobulin genes. Consequently, they lack functional lymphocytes and do not develop cellular or humoral immunity. Therefore, they have emerged as a model for the study of natural resistance (Bancroft and Stevens, 1996). However, some mice gradually develop a low level of T-cell function and some amount of serum immunoglobulin (Ig) ('leaky SCIDs') (see the chapter by Mossmann *et al.*, p. 109ff).

However, also in normal mice the susceptibility to infection and the type of immune response to various antigens are under genetic control. Therefore, great care has to be taken before an observation made or not made in one strain of mice is generalized to other strains. In immunological studies, at least C57BL/6, Balb/c, AKR and Swiss mice should be tested (Table 4). B10 mice may be of great value in evaluating peptide vaccines because of the availability of various H-2 haplotypes based on the same genetic background (Table 5).

For some purposes it may be useful to reduce the effects of LPS. Endotoxin-hyporesponsive C3H/HeJ mice fail to respond normally to endotoxin because their B cells and mononuclear phagocytes are hyporesponsive to the lipid A moiety of LPS.

Recently, the number of available transgenic or gene knockout (GKO) mice has been increasing dramatically, and these mice are most interesting tools in immunological research. It must be kept in mind, however, that certain genetic defects may result in profound changes in anatomy

Table 4. H-2 loci of commonly used mouse strains

Haplotype		K	Ab	Aa	Eb	Ea	D	Thy-1	CD8
A/J	a	k	k	k	k	k	d	2	2
C57BL/6J(B6)	b	b	b	b	b	–	b	2	2
DBA/2(J)	d	d	d	d	d	d	d	2	1
Balb/c(J)	d	d	d	d	d	d	d	2	2
C3H/He(J)	k	k	k	k	k	k	k	2	1
AKR/J	k	k	k	k	k	k	k	1	1
CBA/J	k	k	k	k	k	k	k	2	2
Swiss mice (SJL)	s	s	s	s	s	–	s	2	2

Table 5. H-2 loci of B10 mice

Haplotype		K	Ab	Aa	Eb	Ea	D	Thy-1	CD8
B10.BR	k	k	k	k	k	k	k	2	2
B10.D2	d	d	d	d	d	d	d	2	2
B10.Q	q	q	q	q	q	–	q	2	2
B10.A	a	k	k	k	k	k	d	2	2
B10.S	s	s	s	s	s	–	s	2	2
B10.A (1R)	h1	k	k	k	k	k	b	2	2
B10.A (2R)	h2	k	k	k	k	k	b	2	2
B10.A (3R)	i3	b	b	b	b/k	k	d	2	2
B10.A (4R)	ha	k	k	k	k/b	b	b	2	2
B10.A (5R)	i5	b	b	b	b/k	k	d	2	2
B10.T (6R)	y2	q	q	q	q	–	d	2	2
B10.S (7R)	t2	s	s	s	s	–	d	2	2
B10.S (8R)	as1	k	k	k	k/s	–	s	2	2
B10.S (9R)	t4	s	s	s	s/k	k	d	2	2

and physiology due to secondary disturbances in ontogeny. Therefore, detailed knowledge of these differences is important in order to prevent misinterpretation of data from experimental infections obtained using those mice (for details see the chapter by Mossmann *et al.*, page 109ff). In addition, using these mice studies on natural resistance are more easily performed than are studies on the immune effector phase or immunological memory.

Routes of Infection and Immunization

The course of infection, the host response and the effect of experimental immunomodulation may depend critically on the route of infection or immunization. In addition, as mentioned above, certain virulence factors may be critical only when bacteria are applied mucosally. The following routes of infection may be used (maximum volume applicable):

- Mucosal applications:
  - peroral (300 µl)
  - intranasal (50 µl)
  - intraurinary (50 µl)
  - intravaginal (30 µl)
  - rectal (30 µl)
- Parenteral applications:
  - intradermal (50 µl)
  - subcutaneous (2 ml)
  - intramuscular (50 µl)
  - intraperitoneal (2 ml)
  - intravenous (300 µl)
  - intracranial (30 µl).

Application to mucosal surfaces

Orogastric application

Bacterial suspensions can be administered using a 0.86-mm polyethylene tube connected to a syringe fitted with a 20-gauge ½-inch needle (Fig. 2) or by a feeding needle. Successful oral administration requires thorough knowledge of the anatomical relationships of the oropharynx (Fig. 5), because the oesophageal orifice cannot be observed easily in the living mouse. The feeding needle (18 or 20 gauge) is introduced into the left diastema and gently directed caudally toward the right rami of the mandible. At this point, the mouse usually begins to swallow and the feeding needle can be inserted gently into the oesophagus.

In models of intestinal infection, there is usually much more variation than in models using parenteral infections. Besides reduced accuracy of application, this might be due to variations in the intestinal flora, which can interfere with the infective agent. Furthermore, individual and strain variations of anatomical structures (e.g. the number of Peyer's patches varying between 6 and 12 per mouse) or functional parameters (e.g. stomach acid, amount of food in the stomach, enteral passage) may account for the diversity. The former might be critical for experiments with enteroinvasive bacteria such as *Salmonella* and *Yersinia* species. As these bacteria enter the intestinal wall via M cells that occur only with the FAE overlying Peyer's patch tissue, the number of Peyer's patches correlates with the

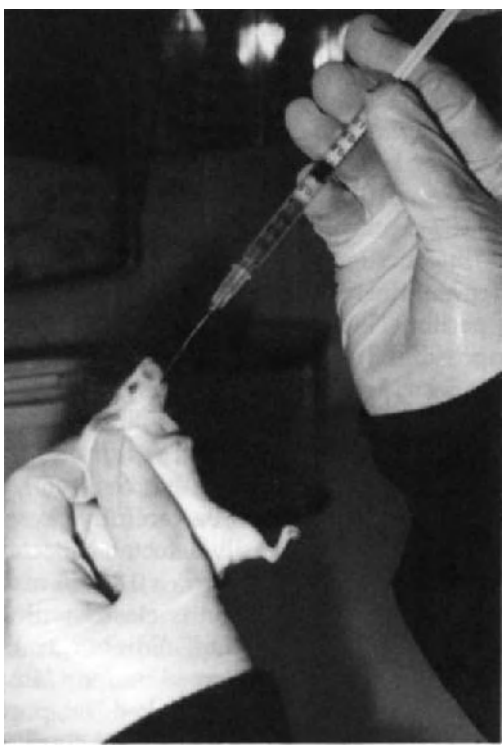


Figure 2. Oral inoculation.

number of M cells and thus with the chance of invasion. Furthermore, it is very important to 'synchronize' mice prior to orogastric infection. For this purpose mice should be starved for 12–18 h to clear the bowel. To avoid coprophagy, which might result in reinfection by excreted bacteria, mice should be kept on grids (Fig. 3).

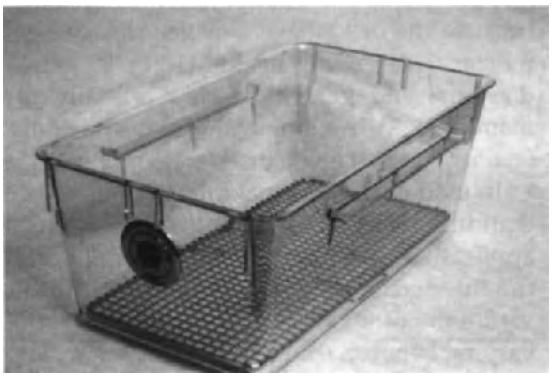


Figure 3. Cage with a grid preventing superinfection by coprophagy.

Ileal-loop model

The ileal-loop model allows the investigation of mechanisms operating in the very early phase of intestinal infections (Autenrieth and Firsching, 1996a). By focusing on a limited part of the intestine, sampling error is reduced. Mice should be starved for 18 h prior to the experiment. Anaesthesia can be accomplished by intraperitoneal injection of 50–70 mg kg^{-1} sodium pentobarbital (Abbott Laboratories, North Chicago, IL, USA). After a deep stage of anaesthesia has been reached (i.e. after about 3–5 min, check by scratching the food pad with a needle) a small midline incision of 1–1.5 cm in length is made down the abdomen in order to reveal the small intestine (Plate 3). Cutting into superficial abdominal or peritoneal vessels must be avoided as bleeding makes proper working impossible. To form a loop, two ligations are made in the ileum, leaving a 3- to 5-cm gap in between. This ensures that the loop contains a Peyer's patch if binding to a Peyer's patch or invasion of the Peyer's patch by bacteria is desired (e.g. *Esch. coli, Salmonella typhimurium, Shigella flexneri, Yersinia enterocolitica, Campylobacter jejuni*). The blood supply to the small intestine should always be carefully preserved. Vessels at the intestine and peritoneum are easily detectable. Bacterial suspension (0.1 ml) can be injected into the ileal loop via a 0.4-mm needle. Return the small intestine to the bowel. The incision is closed with a suture or two (e.g. prolene monofilament, Ethicon Ltd., Edinburgh, UK). During the whole procedure, mice should be warmed under a lamp, and the intestine should be kept wet with a towel soaked in prewarmed sterile saline 37°C. Disinfection is usually not required since a typical ileal loop experiment usually lasts only for a few hours.

Intranasal application

Infection of the respiratory tract can be achieved either surgically or non-invasively. Whenever possible, excess manipulations should be avoided, thus reducing stress and increasing reliability and reproducibility of the model.

Non-surgical methods. The murine trachea has a diameter of only 1–1.5 mm and the distance between nose and the lower parts of the lung is approximately 2.5–3 cm in adult mice. The inoculum size (50 µl) is thus a critical factor to the success of intranasal delivery. Inocula less than 10 µl are more likely to stick to the upper respiratory tract and thus may not reach the lungs. Another important parameter is the depth of anaesthesia. The mouse has to be anaesthetized deeply enough to suppress the swallowing reflex so that the inoculum actually reaches the lung and does not end up in the stomach or is expectorated by the mouse. On the other hand, delivering a bolus into the trachea induces a vagal response resulting in a reduced heart rate. If anaesthesia is too deep, cardiac arrest may result. Anaesthesia for this kind of manipulation should be performed by intraperitoneal administration of ketamine hydrochloride and xylacine (Rompun) together.

The dose depends on the weight and strain of mice used. For example, CD-1 (ICR) mice may require doses almost 1.5 times higher than for C3H/HeN or C3H/HeJ mice. Usually, ketamine hydrochloride and xylacine are diluted in sterile, pyrogen-free PBS in a ketamine hydrochloride/xylacine ratio of 20:1, resulting in a dose of 120 mg kg^{-1} of ketamine hydrochloride and 6 mg kg^{-1} of xylacine. After reaching deep anaesthesia, which occurs usually within 5–10 min, mice are held upright and the inoculum is delivered by means of a pipette into one nostril (Fig. 4). This ensures that the mouse can continue to breath through the contralateral nostril. The pipette tip should be placed about 1–2 mm apart from the nostril in order to obtain fine droplets of about 0.5–1 mm diameter which easily enter the nose and do not cloak major airways. The whole procedure should take no more than 1 min. Hyperventilation is a welcome side-effect, since it contributes to an even distribution of the inoculum throughout the lungs. The mouse should be held upright for another minute or two to ensure entry of the inoculum into the lungs. Then, the animal is placed back in its cage such that the head and thorax are slightly elevated. After 30–60 min, the animal usually awakes from anaesthesia. Using this approach, approximately 75–85% of the inoculum will end up in the lung. Plate 2 shows a lung after inoculation of 50 µl india ink to depict the distribution of the inoculum in the lungs. It must be stressed that the lungs cannot be inoculated separately by any method. As can also be seen in Plate 2, there is an uneven distribution of ink in the alveoli, despite homogeneous coating of the major bronchi. This means that the pattern of infection will be patchy, which is of consequence for the subsequent analysis of the tissue response.

Another way to administer inocula into the lungs of mice is by intubation using a small feeding tube (not more than 26 gauge) which is inserted slowly into the mouth (Fig. 5). However, this requires manipulation and

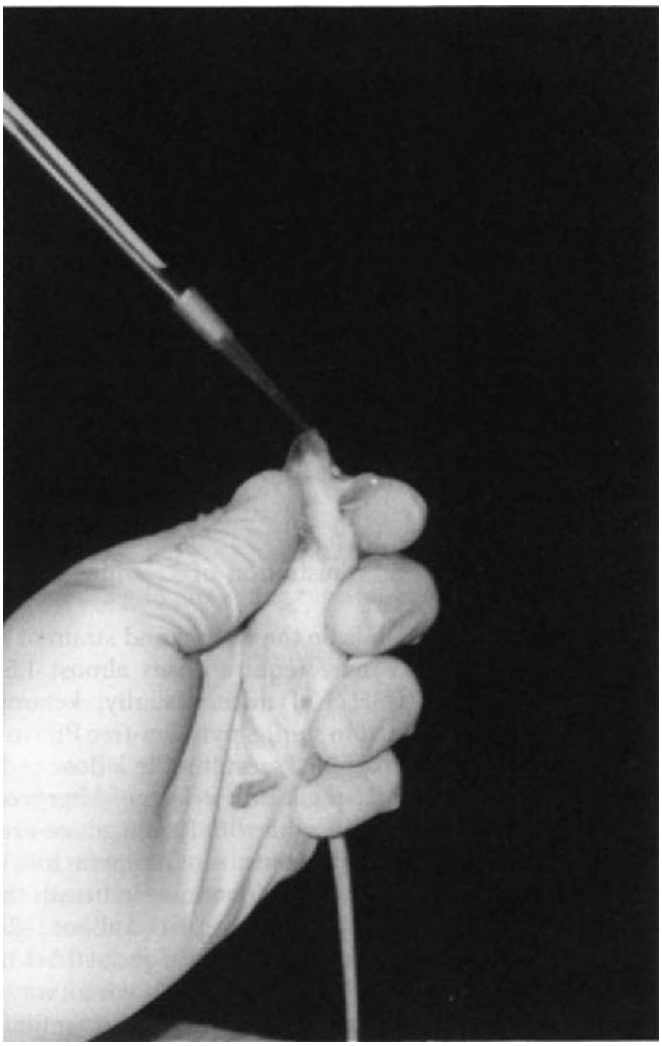


Figure 4. Intranasal inoculation.

bears the danger of injury. In addition, it takes more time to apply inocula by this technique. Thus, if large numbers of mice have to be processed, the intranasal route is preferred.

Surgical methods. After deep anaesthesia (see above) a small incision is made at the midline of the neck, directly above the trachea, not more than 1–1.5 cm in length. Only the skin should be cut, in order to avoid damage to the underlying thyroid gland. This organ as well as the muscles covering the trachea are prepared using atraumatic techniques and by carefully avoiding incision of blood vessels. The trachea is then exposed and a small cut (not more than 1–2 mm) is made between two cartilages of the

trachea (Fig. 15). Either a small feeding tube (26 gauge or less) with a blunted end or a Teflon catheter (e.g. Abbocath) is inserted and the desired volume applied to the lungs. Again, even with this method, it is impossible to incubate one lung selectively. The trachea should be closed with one suture. It is not necessary to close the muscles over the trachea; however, the skin should be closed with a suture.

Aerosols. A detailed description of infection by aerosols is given in the chapter by Roberts *et al.* (p. 389ff).

Jrinary tract application

For urinary tract infection, mice are anaesthetized as described above and a polyethylene catheter (2.5 cm long, 0.61 mm outer diameter; Kebo Grave, Sweden) is inserted via the urethra into the bladder (Fig. 6). Prior to insertion of the catheter, the bladder should be emptied by gentle compression of the abdomen. The catheter should be fitted to a needle (0.4 × 0.22 mm gauge) on a 1-ml tuberculin syringe and up to 50 µl of a bacterial suspension can be administered.

'aginal and rectal application

For vaginal and rectal administration of bacteria, mice should be anaesthetized. An inoculum of about 30 µl can then be introduced gently into the rectum or vagina using a 23-gauge needle with the end blunted with solder or a yellow tip (Fig. 6). After vaginal or rectal administration mice should be positioned with the vagina or rectum facing upwards for 30–60

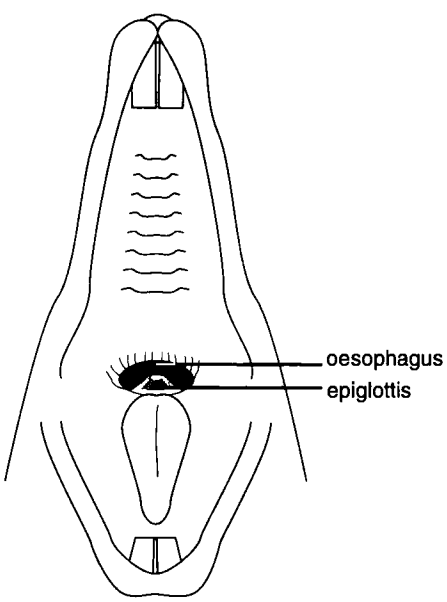


Figure 5. Murine oropharynx.

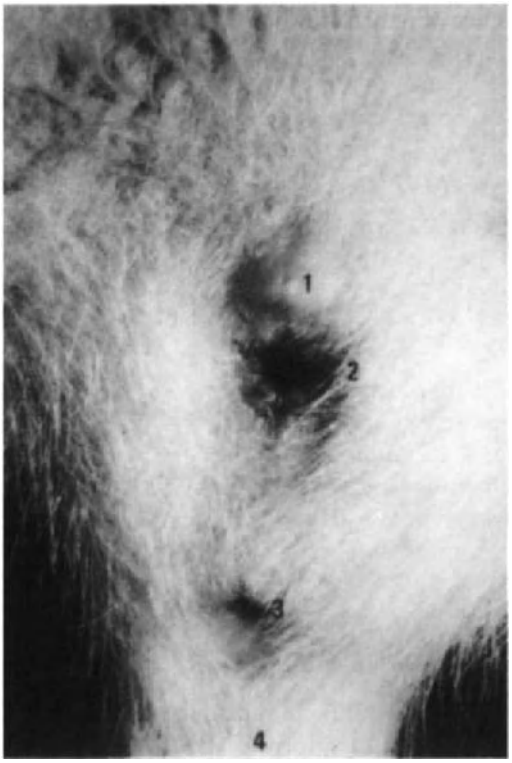


Figure 6. Rodent rectum and introitus vaginalis: 1, ostium urethrae externum; 2, ostium vaginae, 3, anus; 4, radix caudae (Olds and Olds, 1979).

min to reduce leakage of the inoculum. Alternatively, a swab soaked with the inoculum is left in the vagina for 20 min. For vaginal infections and immunizations, it is important to consider the oestrous status of the animals (Hopkins *et al.*, 1995). Mice at the late metestrus or diestrus exhibit stronger immune responses upon vaginal immunization. Therefore, female mice may receive 2.5 mg medroxyprogestrone (Depo Provera, Upjohn, Puws, Belgium) per dose subcutaneously in 100 µl PBS 7 days prior to intravaginal inoculation.

Routes of infection bypassing mucosal surfaces

If an infection cannot be achieved via the mucosal route, it may be helpful to bypass mucosal surfaces by parenteral injection.

Subcutaneous and intradermal injection

The subcutaneous route of administration may be utilized when prolonged release of a relatively large inoculum is required. Subcutaneous injections (1 ml) can be made into the loose skin over the flank using a 26-

to 30-gauge, 0.5- to 1-inch needle. The needle should be inserted into the skin 0.25-inch caudal to the injection site, and then advanced through the subcutaneous tissues to the injection site in order to minimize leakage of the injected material.

Intradermal injection into the volar aspect of the hind foot pad is often used to investigate the immune response of the skin and to elicit secondary immunological responses such as the Arthus reaction or DTH (Fig. 7). A 30-gauge needle is inserted between pulvini and advanced just under the surface of the skin. Correct injection of up to 50 µl results in a pale bulla.

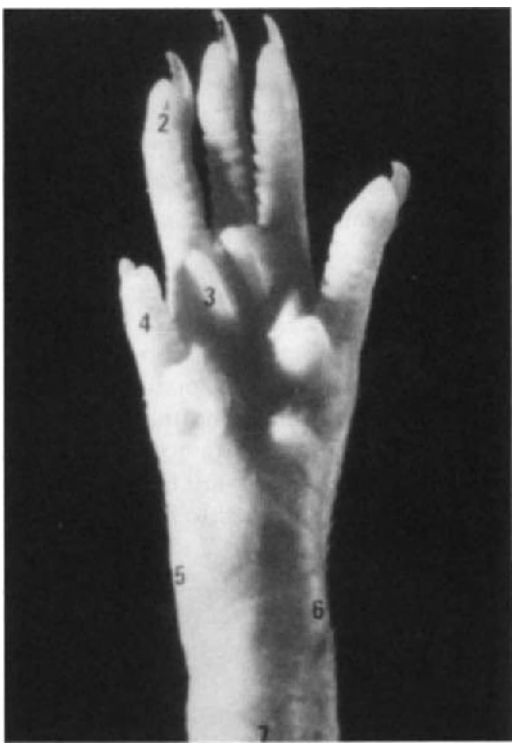


Figure 7. Rodent hind foot pad: 1, ungicula; 2, pulvini phalangici; 3, pulvini metatarsales; 4, hallux; 5, median side; 6, lateral side; 7, ankle (Olds and Olds, 1979).

ıtramuscular injection

Intramuscular injection should usually be avoided in the mouse because of the small muscle mass. The rate of absorption of aqueous solutions is similar following intramuscular and subcutaneous injections, and so the latter is preferred. If necessary, intramuscular injections of up to 50 µl may be made into the anterolateral thigh muscles (quadriceps femoris group) using a 22- to 26-gauge 0.25-inch needle. The needle should be directed away from the femur and sciatic nerve (Fig. 8).

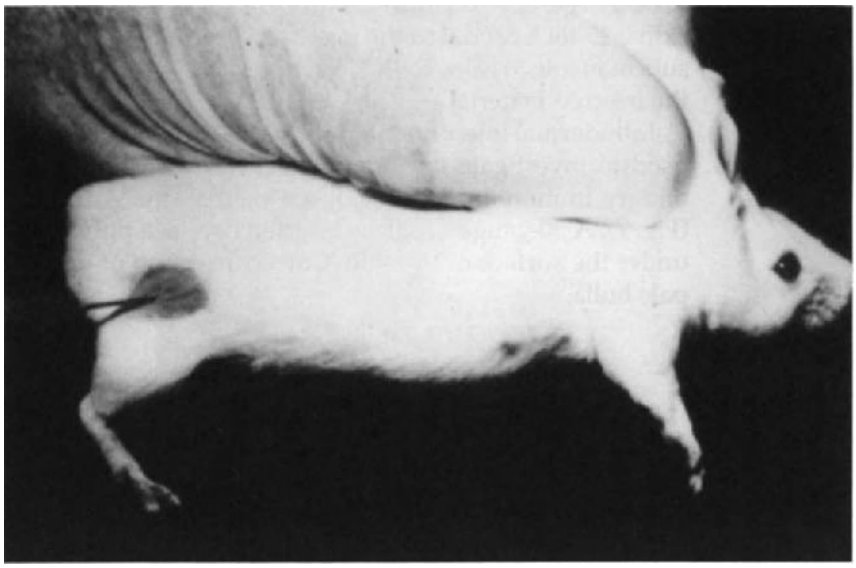


Figure 8. Intramuscular injection (Versteeg, 1985).

Intraperitoneal injection

Intraperitoneal injection is the most convenient and simple technique to apply volumes up to 2 ml. To avoid puncture of the stomach, spleen or liver, intraperitoneal injections are made into the lower right quadrant of the ventral abdomen (Fig. 9). The mouse is restrained, and the handler's wrist is rotated until the mouse's head and body are tilted in a downward direction, allowing the mouse's abdominal viscera to shift cranially. The needle (23- to 26-gauge, 0.25-

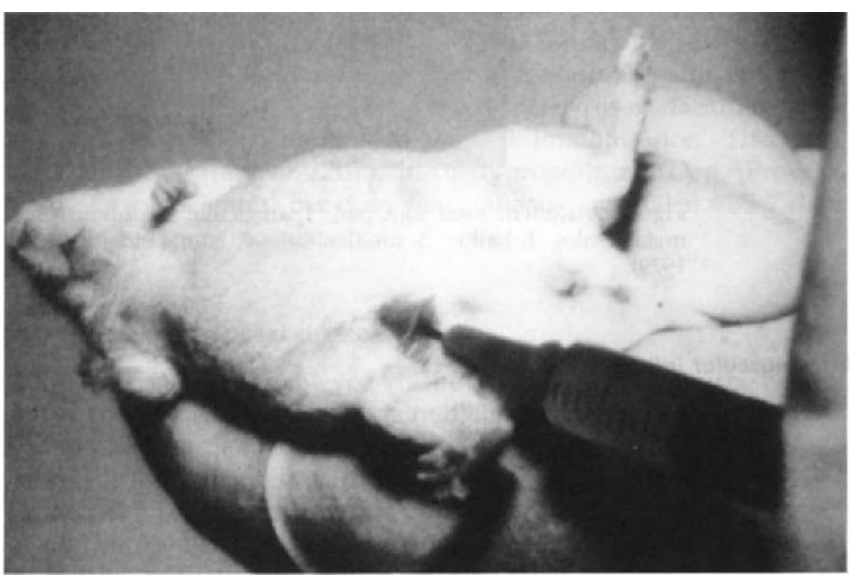


Figure 9. Intraperitoneal injection (Versteeg, 1985).

to 0.5-inch) is then inserted through the skin, slightly medial to the flank and cranial to the inguinal canal, advanced cranially through subcutaneous tissue for 2–3 mm, and then inserted through the abdominal muscles. The needle and syringe should be held parallel to the mouse's vertebral column in order to avoid accidental retroperitoneal or intrarenal injection.

Intravenous injection

The lateral or dorsal tail veins are the usual sites for intravenous injection in mice (Fig. 10). Tail vein injection is easier if the veins are dilated by gently warming the mouse for 5–15 min with a 40–100 W light bulb. Lateral tail veins are visualized as thin red-blue lines. The needles used are 26- to 30-gauge, 0.25- to 0.5-inch.

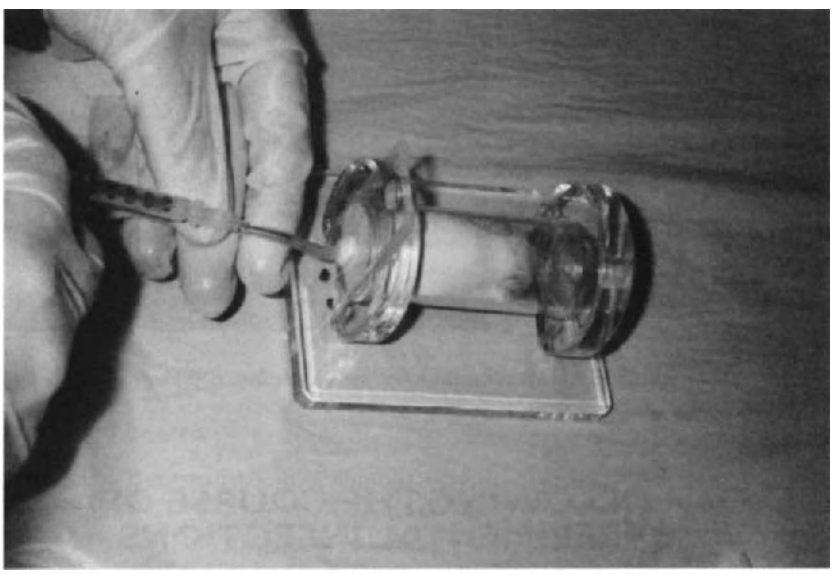


Figure 10. Intravenous injection.

Intracranial injection

Intracranial injection has been used to establish *in vivo* models of infections with neurotropic viruses, but may also be used for bacterial infections. This route may be helpful in investigating whether immunity induced in the periphery is expressed in the central nervous system as well. Mice are anaesthetized as described above, and the scalp is disinfected with 70% ethanol. The needle (22- to 30-gauge) is inserted through the skin, over the midsection of the parietal bone slightly lateral to the central suture; this avoids puncture of the sagittal or transverse venous sinuses (Fig. 11). The needle is gently rotated until the bone is penetrated. The needle is then advanced to a depth of 1–4 mm, depending on the size of the mouse. Approximately 15–30 µl can be injected intracranially. Solutions injected intracranially should be at 37°C. After injection, the mice should be kept warm to reduce the possibility of shock.

Figure 11. Intracranial injection (Versteeg, 1985).

◆◆◆◆◆◆ FOLLOWING THE COURSE OF EXPERIMENTAL INFECTIONS

Morbidity and Mortality

Body weight

Usually, animals are infected and then observed over a certain period of time, the end-point being survival. Acquiring information in between these times may be difficult. Therefore, careful observation may provide important clues about the course of an infection and the best suited time points for more detailed investigation by invasive methods. After infection, mice may show non-specific signs of illness, such as inactivity, rough fur, conjunctivitis, dyspnoea or diarrhoea. Rolling, circling or paresis may be signs of neurological disease. In some cases mice may develop skin lesions or swelling. However, the onset of illness is seldom so obvious that time to illness would be a reliable parameter for measuring the kinetics of the infectious process. Moreover, quantification of severity is very difficult. One quantifiable parameter that can be observed during the course of infection is loss of body weight. Weighing the animals is a simple way to obtain this information. In bacterial infections in particular,

animal weight may correlate not only with final outcome, but also with histopathology and bacterial organ load. Thus, measuring body weight daily or at even closer time points may be a reliable non-invasive method of acquiring the first information about the course of an infection.

LD_{50} and time to death

Outcome and course of infection are critically influenced by the size of the inoculum. If a lethal challenge is used, the major parameters that can be studied are lethality and time to death. The estimation of the dose of a pathogen resulting in the death of a given percentage of animals is a global procedure – it can be used to compare both the virulence of different pathogens, and also a single pathogen administered by different routes or expressing different virulence factors in the case of isogenic mutants. The most commonly used end-point is the LD_{50}, which denotes the amount of pathogen necessary to kill 50% of the animals. To determine the LD_{50}, mice are infected with various doses of bacteria ranging from 10^1 to 10^9 cfu per animal. Mice are then observed over a period of time, ranging from days to several months depending on the infective agent. The LD_{50} is calculated according to a method published in the late 1930s (Reed and Muench, 1938). Most often, 5–10 animals per dose of pathogen have to be used for calculation (see p. 355).

Methods Requiring Sacrifice of Animals

Bacterial load in infected organs

Quantitative determination of the bacterial load in various organs allows a more detailed assessment of the infectious process. Furthermore, less animals are required for a definite result than for determination of LD_{50}.

The determination of numbers of bacteria per infected organ is the most direct parameter for following the course of an infection and defining protective host responses. It has to be kept in mind, however, that morbidity and lethality are the result of both bacterial replication and the inflammatory host response. Hence to obtain a complete picture, histological studies must be done in parallel.

A major disadvantage of the procedure is that mice have to be killed in order to obtain this information. However, recent work has demonstrated that bioluminescence may be applied to *in vivo* analysis of bacterial replication. Bacteria are transformed with a plasmid conferring constitutive expression of bacterial luciferase. Detection of photons transmitted through tissues of infected mice allows localization of bacteria by real time and non-invasive monitoring (Contag *et al.*, 1995).

It is worth mentioning that the use of high lethal doses of bacteria may result in the infection of unusual target organs (e.g. the brain) or host cells. Therefore, another advantage of the quantification of viable bacteria in infected organs is the ability to characterize sublethal infections. In a typical experiment mice are infected and a certain number of animals out of

this group is killed by CO_2 and cervical dislocation at various time points after infection.

In order to obtain maximum information, organs of at least three mice may be cut into three equal pieces

- one for the determination of bacterial load
- one for histological analysis
- one for cellular and/or molecular analysis, such as the determination of soluble mediators or cell functions by cell culture studies.

However, whenever unequal distribution of the infection is expected, as in lung or gut after nasal or oral infection, respectively, the entire organ should be used for one single type of analysis (e.g. only cfu determination) in order to minimize sampling errors.

The basic method for determining bacterial load is easy to perform; the only difficulty being sterile preparation of certain organs such as Peyer's patches. Organs are removed aseptically (Plate 3) and parts or the whole organ are transferred, after weighing in a sterile Petri dish, into homogenizer tubes containing 5–10 ml sterile PBS (Fig. 12). Organs are dispersed for 1 min using a motorized homogenizer placed in a safety cabinet (Fig. 13). The homogenate is subsequently stored on ice until serial dilutions (1:10) in sterile PBS are plated on suitable and absorbent (predried) agar (e.g. TS or BHI agar). Bacterial colonies are counted after an appropriate incubation period, depending on the bacterium under investigation, and numbers may be normalized for organ/tissue weight.

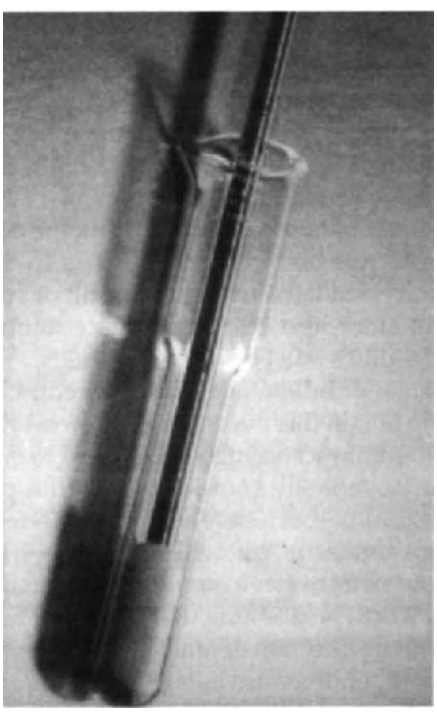


Figure 12. Glass tube with Teflon pistil (Versteeg, 1985).

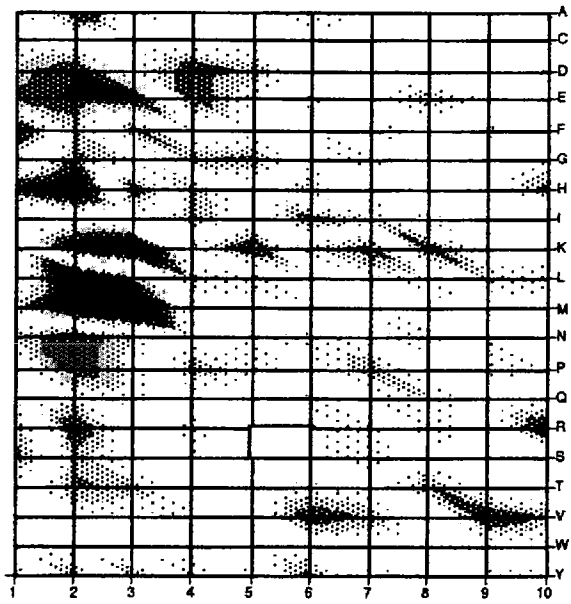


Plate 1. Visual representation of the MHC binding matrix for HLA A-0201 illustrating the concept that selected amino acid residues can promote binding (for example, L or M in position 2), while others may inhibit binding (for example, P in position 2). The peptide epitope prediction algorithm EpiMatrix determines an estimated binding potential for any given peptide by comparing its score on this matrix of possibilities and the EpiMatrix scores of known ligands for that MHC.

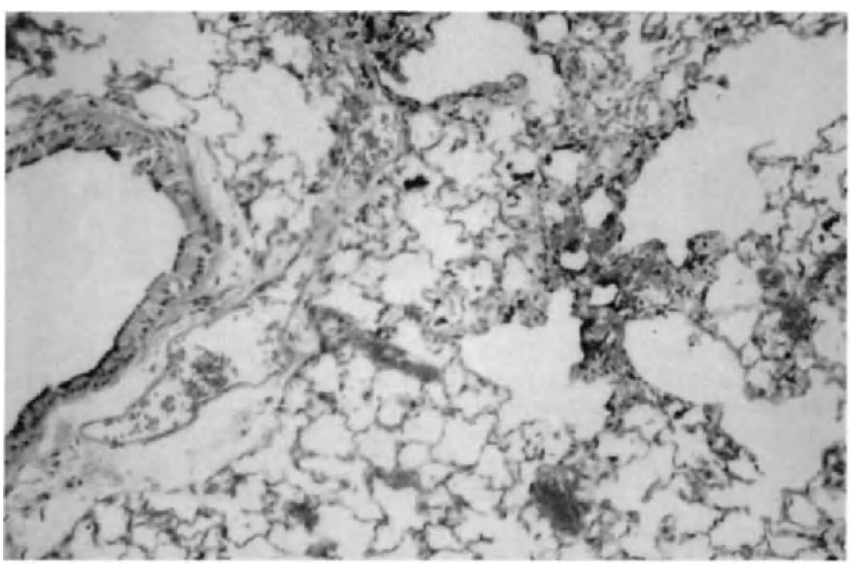


Plate 2. Murine lung showing the distribution of intranasally administered India ink. H & E, × 320

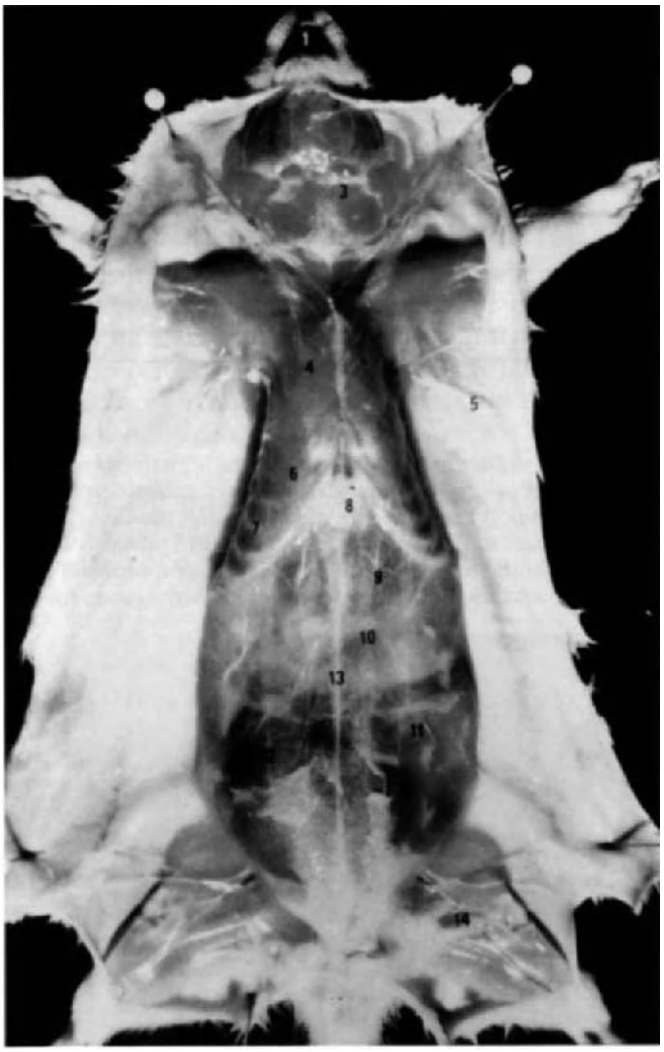


Plate 3. Rodent sites: 1, rima oris; 2, masseter muscle; 3, Lc. mandibulare, Gl. mandibularis and Gl. parotis; 4, pectoral muscle; 5, cutaneous nerves; 6, thorax; 7, ribs; 8, xiphoid cartilage; 9, liver; 10, stomach; 11, intestine; 12, caecum; 13, linea alba; 14, saphenus nerve, artery and vein (Olds and Olds, 1979).

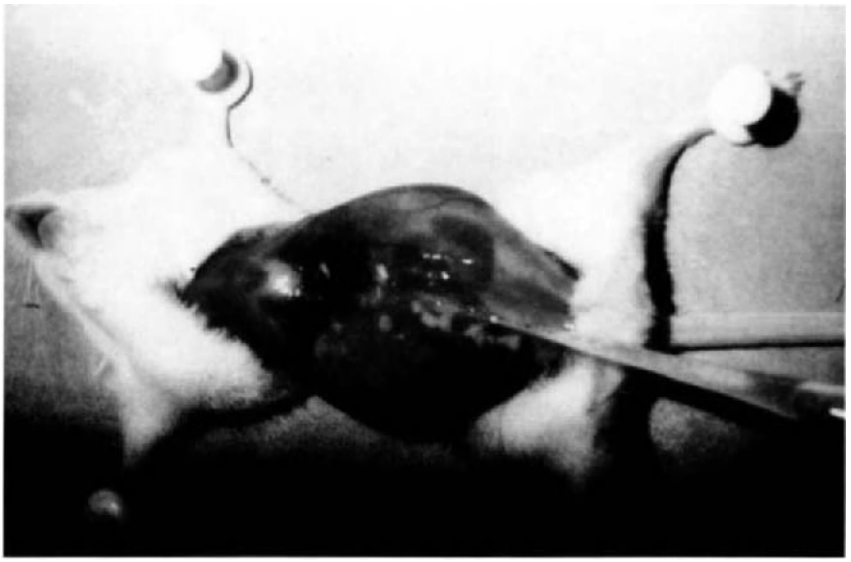


Plate 4. Lavage of the peritoneal cavity (Versteeg, 1985).

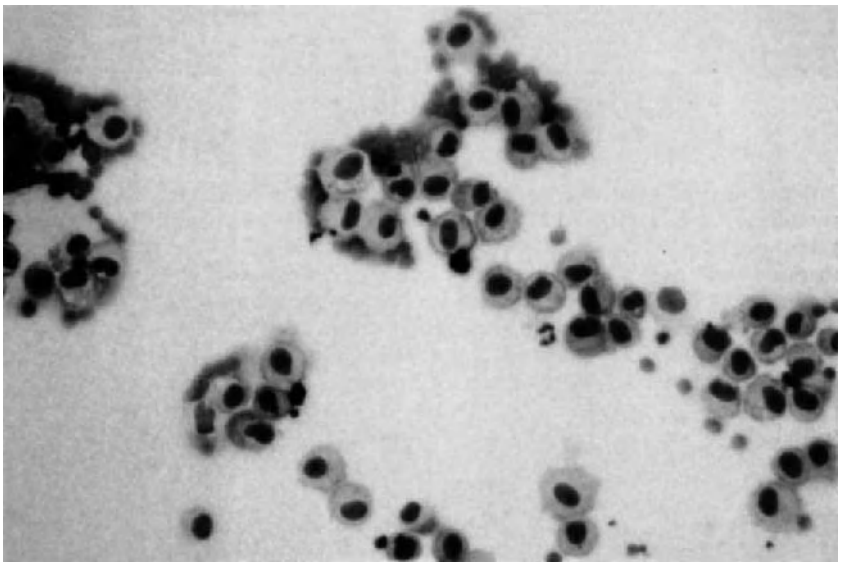
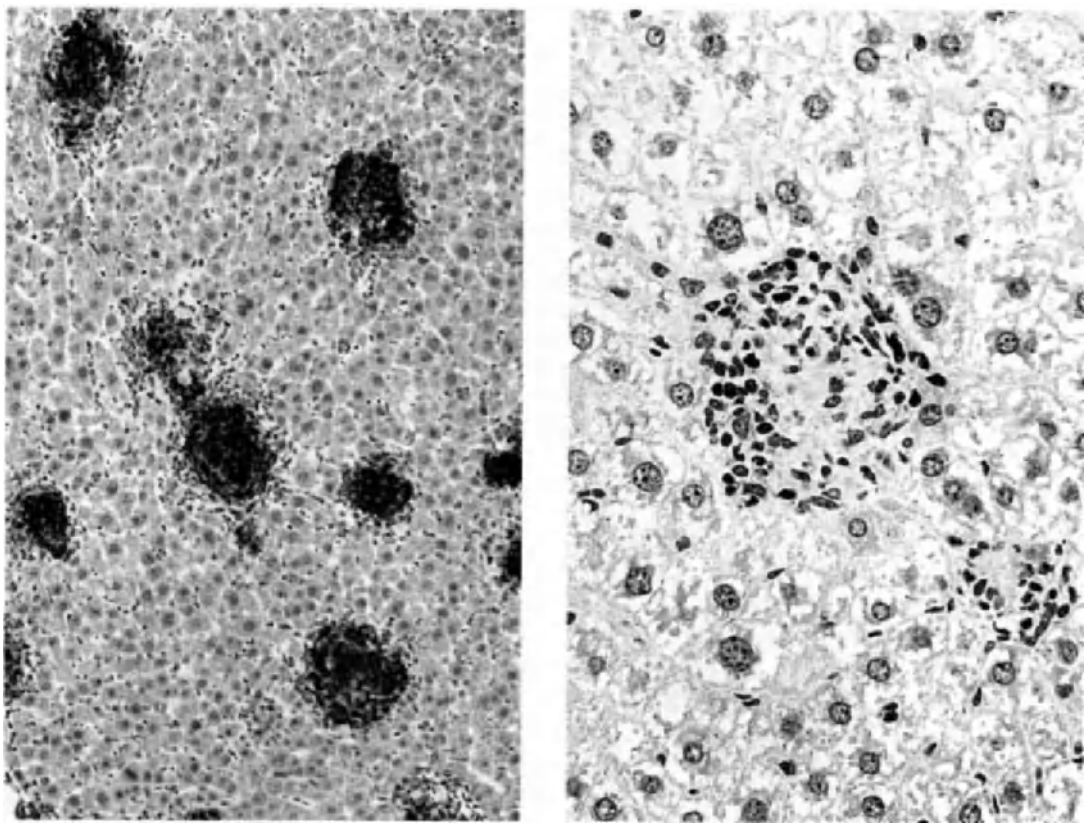


Plate 5. Typical cellular composition of bronchoalveolar fluid. Diff-Quick stain, ×640.



(A) (B)

Plate 6. (A) Expression of inducible nitric oxide synthase (iNOS) in granulomas of C57BL/6 mice intravenously infected with 2×10^5 cfu *Mycobacterium avium* 6 weeks previously. As primary antibody, a polyclonal rabbit–anti-iNOS antiserum (Genzyme) was used at a 1:1000 dilution. Pressure cooking of paraffin-embedded sections was performed for 1 min. (B) Expression of Ki-67 (proliferation-associated) antigen in granulomas of C57BL/6 mice intravenously infected with 2×10^5 cfu *M. avium* 4 weeks previously. As primary antibody, a polyclonal rabbit–anti-mouse-Ki-67-equivalent antiserum (kindly provided by J. Gerdes, Borstel) was used at a 1:1000 dilution. Microwaving of paraffin-embedded sections was performed five consecutive times for 5 min.

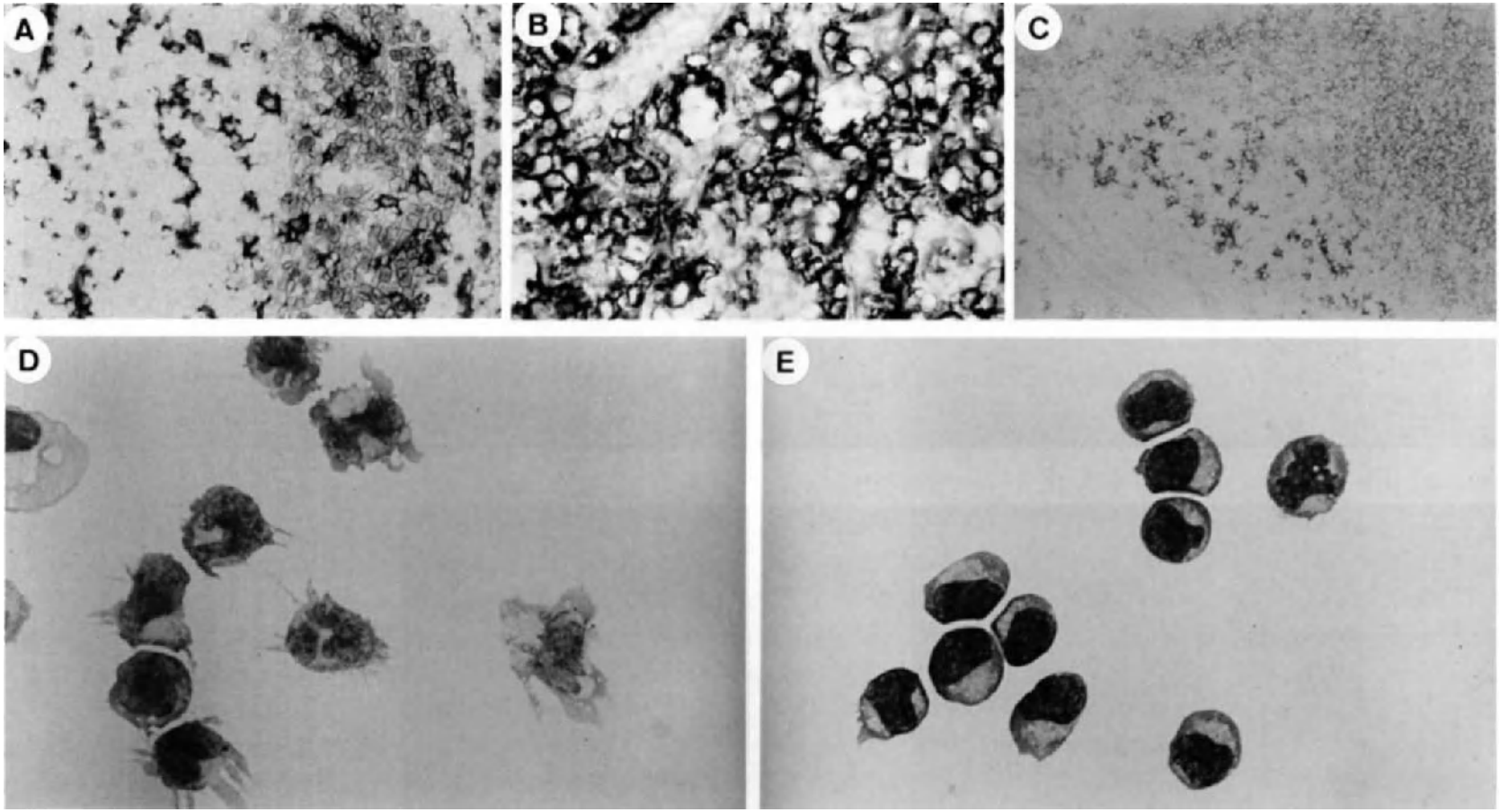


Plate 7. *In situ* identification of IDC, GCDC and $CD11c^-$ precursors in tonsils. Immunostaining was performed on sections of tonsils. (A) Anti-CD11c (red) and anti-Ki67 (blue nuclear) staining shows that $CD4^+CD11c^+$ GCDC are evenly distributed both in the dark zone (packed with $Ki67^+$ centroblasts) and in the light zone of the germinal centre. (B) Double staining with anti-CD3 (blue), anti-CD11c (blue) and anti-CD4 (red) shows red $CD4^+CD11c^-$ $CD3^-$ DC precursors (plasmacytoid cells) localized in the T-cell area (within or around HEV), but not in the germinal centre. (C) Staining with anti-CD40 shows IDC expressing high levels of CD40. (D) May–Grünwald–Giemsa of freshly isolated GCDC. (E) May–Grünwald–Giemsa of freshly isolated $CD4^+CD11c^-$ precursors. Magnification: (A–C) ×200; (D, E) ×400.

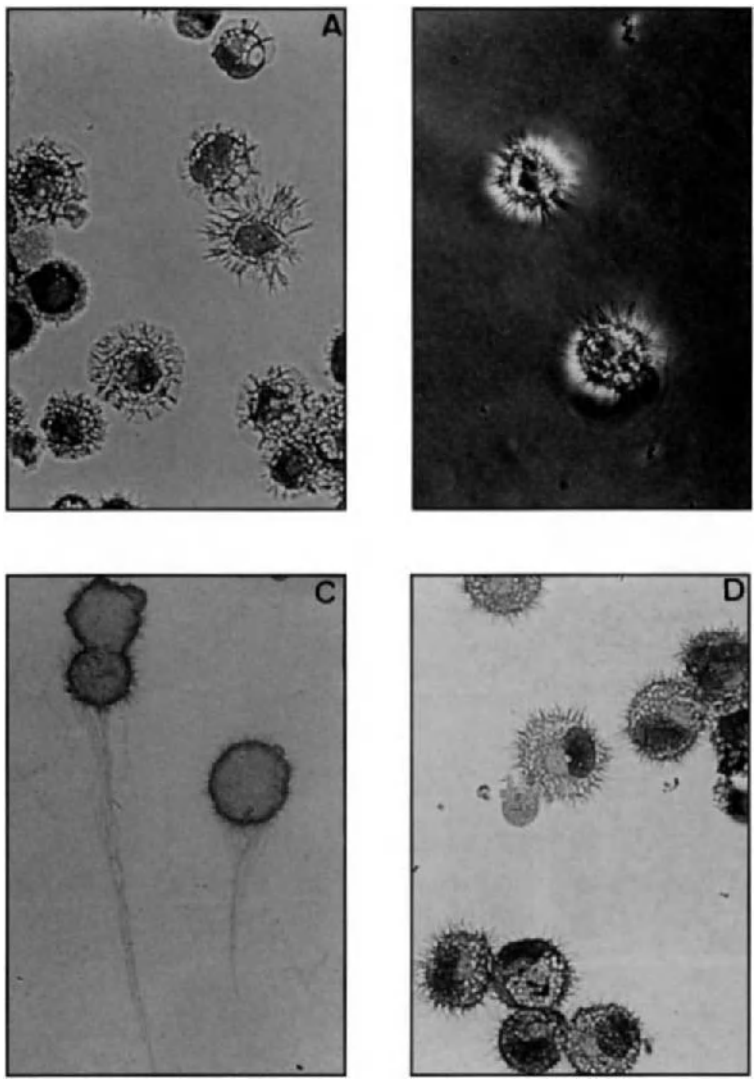
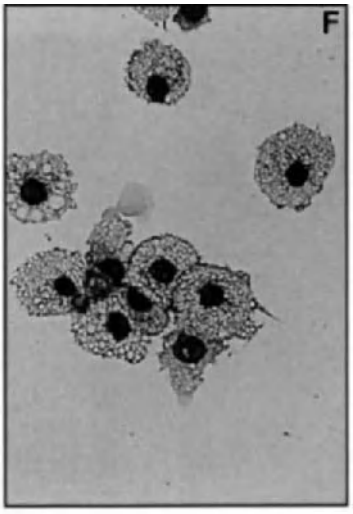
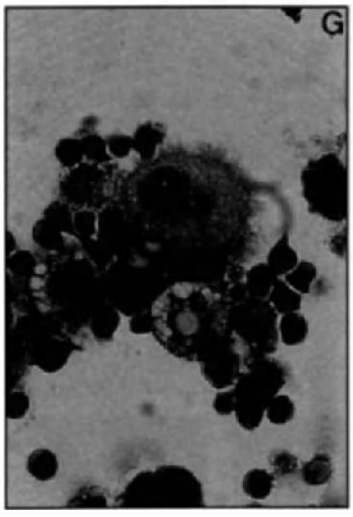
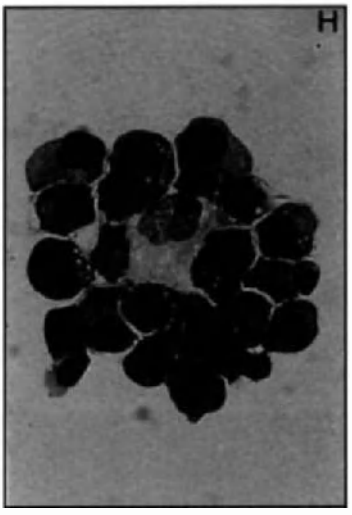


Plate 8. Morphology of cells generated from $CD34^+$ HPC in response to GM-CSF plus TNFα. $CD34^+$ HPCs were cultured for 12 days with GM-CSF plus TNFα (A) May–Grünwald–Giemsa staining of total population. (B) Phase contrast microscopy of total population. (C) HLA-DR immunostaining of total population. (D) Day 12 $CD1a^+$ derived DCs (see Fig. 4, Chapter III.1). (E) Day 12 $CD14^+$ derived DC (see Fig. 4, Chapter III.1). (F) $CD14^+$ precursors cultured in presence of M-CSF from days 6 to 12. (G,H) Co-culture of GM-CSF plus TNFα generated DCs and allogeneic $CD4^+$ T cells at 24 h (G) and 96 h (H). (I) CD40-activated B cells in close contact with GM-CSF plus TNFα generated DCs. Magnification: (A–F) ×400; (G–I) ×800.







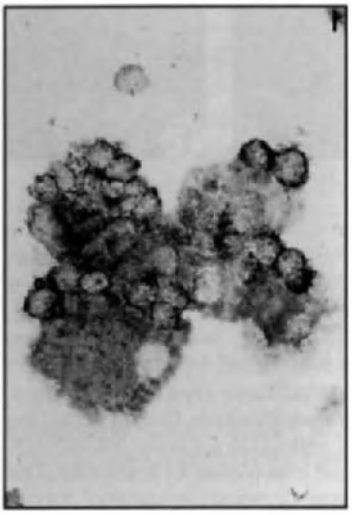


Plate 8. (cont.)

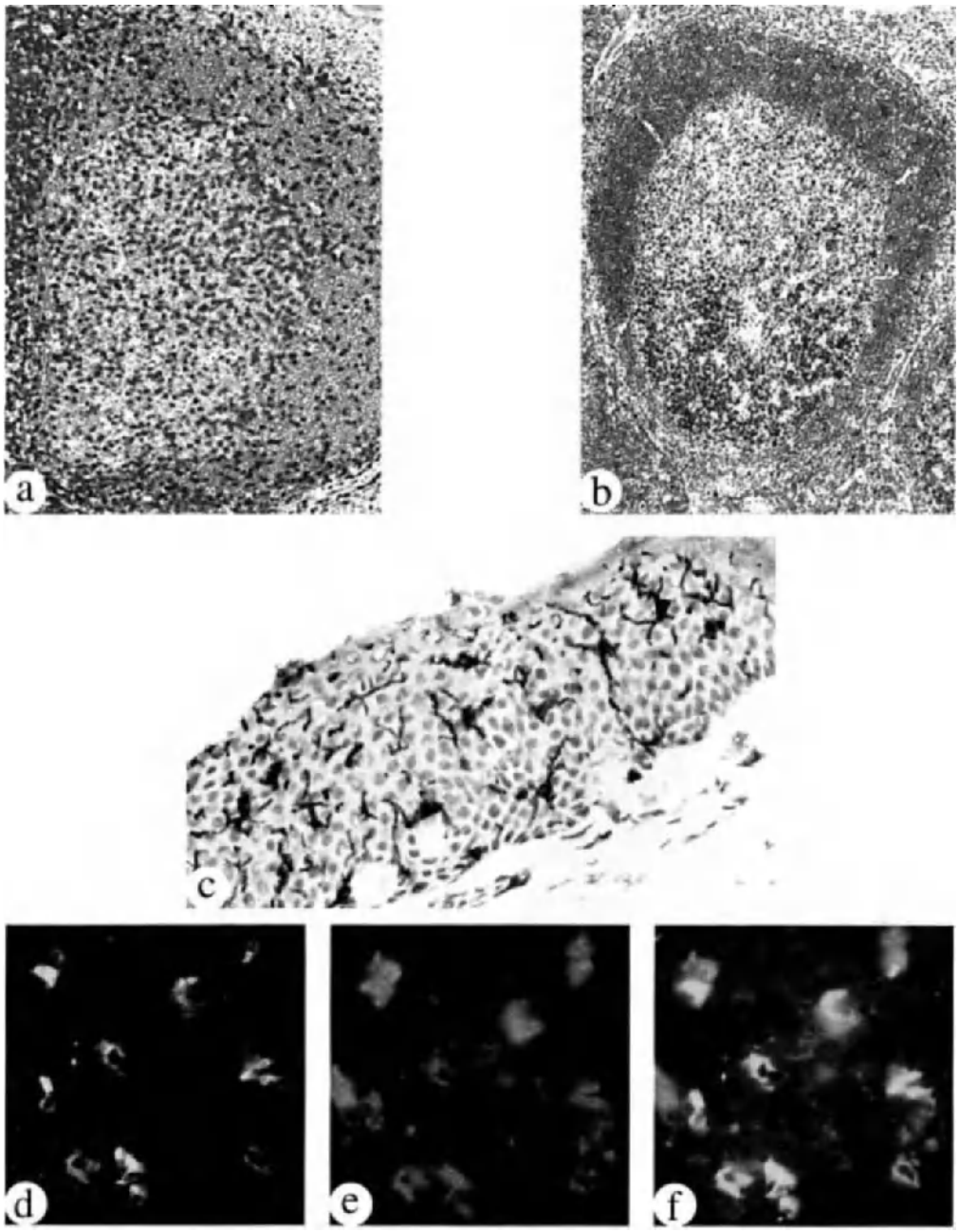


Plate 9. (a, b) A frozen section of a tonsillar germinal centre stained using the APAAP staining procedure: (a) detection of $CD3^+$ T cells using the monoclonal anti-CD3 antibody Leu4 (Becton Dickinson, Heidelberg, Germany); (b) detection of proliferating cells using the Ki-67 antibody, which detects an antigen associated with cell proliferation. (c) Demonstration of $CD1a^+$ Langerhans cells in the epidermis using the indirect immunoperoxidase staining technique (antibody Okt6, Ortho Diagnostic Systems, Neckargemünd, Germany). (d–f) Co-localization of CD26 and γ-interferon (IFN-γ) in a tuberculoid leprosy granuloma using the double immunofluorescence technique. (d) Expression of IFN-γ in green fluorescence is detected by a FITC-conjugated anti-IFN-γ antibody (clone GZ 4, Thomae Research Laboratories, Biberach, Germany). (e) Expression of CD26 in red fluorescence is detected by indirect staining with the anti-CD26 antibody MIB-DS2/7 (this laboratory). Bound antibody is detected with a rhodamine-labelled goat anti-mouse Ig antiserum (Dianova, Hamburg, Germany). (f) A double exposure for green and red fluorescence demonstrates areas of double-positive staining with yellow fluorescence, showing that all IFN-γ positive cells are also positive for CD26.

Figure 13. Potter homogenizer in a biosafety cabinet.

Microbial load in Peyer's patches

To remove Peyer's patches, the whole small intestine (Fig. 14) is excised and put into a Petri dish containing cold sterile PBS. The small intestine is then washed extensively with 10–20 ml cold buffer to remove intestinal contents and bacteria associated with the mucosal surface. Alternatively, gentamicin (10 μg ml^{-1}) may be added to kill bacteria located in the lumen of the intestine. Finally, Peyer's patches are excised by carefully cutting the gut longitudinally. As Peyer's patches are contaminated at their mucosal surface with bacteria from the intestinal flora, which cannot be removed completely by washing, the homogenates should be plated on selective agar media in order to suppress growth of contaminating bacteria (e.g. cefsulodin–irgasan–novobiocin agar for *Yersiniae* spp., SS agar for *Salmonella* spp. or *Shigella* spp).

Description and quantification of tissue lesions and inflammatory host responses

There is no simple way to quantify the degree of tissue destruction and inflammation *in vivo*. In infections afflicting the liver the determination of serum levels of hepatic enzymes may reveal some information, although we do not recommend their use.

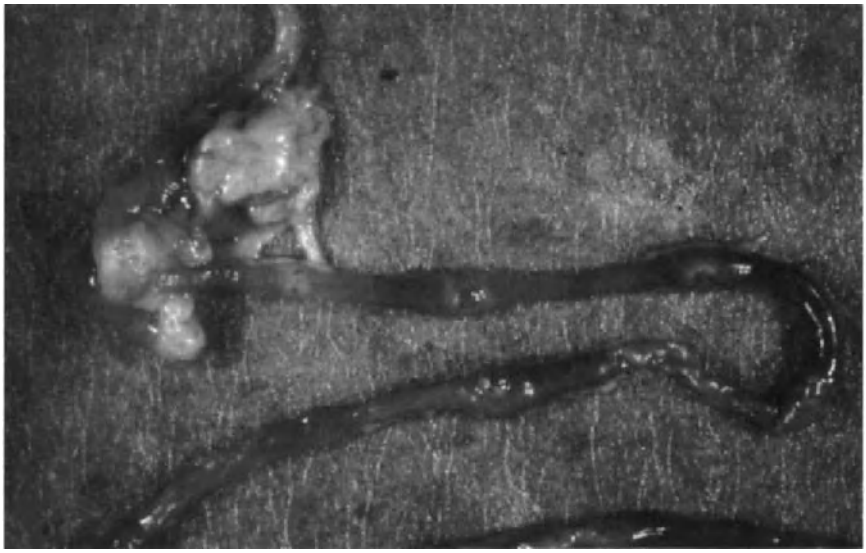


Figure 14. Peyer's patches in rodent gut.

Alanine aminotransferase (ALAT) is a leakage enzyme that is frequently used for the purpose of assessing hepatic injury. However, ALAT is not specific for liver. Therefore, its activity in serum should be considered together with other enzymatic data, such as aspartate aminotransferase (ASAT) and alkaline phosphatase (AP). In addition, it has to be kept in mind that the serum activities of leakage enzymes may decline in the presence of ongoing tissue destruction due to disturbances in the synthesis of the enzymes as the injurious process intensifies. An inflammatory parameter correlating well with the severeness of infection is IL-6. This may easily be determined in serum and organ homogenates by ELISA or bioassay (see the chapter by Fujihashi *et al.*, page 257ff). The most reliable data, however, can be obtained by histological evaluation.

Another aspect that may be of interest in models of infection is to determine the host cell of intracellular bacteria *in vivo*. Careful histology is the only way to do this. However, only high numbers of bacteria can be detected by microscopy (usually $>10^6$ cfu per organ). In some cases only the suppression of host defence mechanisms (e.g. by γ-irradiation, corticosteroids, IL-10) will reveal the permissive host cell.

Histopathological examination

The detailed description of histopathological techniques is outside the scope of this chapter. For details, see Bancroft and Stevens (1996) and the chapter by Ehlers and Seitzer (p. 365ff). However, some aspects that may be helpful in planning an experiment are mentioned below.

Tissue fixation and embedding in plastic. The most powerful histological evaluation is possible by performing semi-thin sections from plastic embedded tissue and staining with haematoxylin and eosin or Toluidine Blue. Bacteria can be visualized by Gram stain, Ziehl–Neelsen stain, Dieterle stain or by Toluidine Blue, which is one of the advantages of the latter stain. Staining procedures and, more importantly, the interpretation of the slides, should be done in collaboration with an experienced pathologist. However, tissue preparation and fixation is decisive for the quality of histological slides and will therefore be described here. It is influenced by pH, temperature, osmolality, the concentration of the fixative and the duration of the process, as well as the size of the sample. Fixation for more than 24 h should be avoided.

Usually, tissues are removed, cut into small pieces (5 × 5 × 5 mm), put into cassettes and immediately immersed in 10% neutral formaldehyde containing 0.1 M sodium cacodylate, or 2% formaldehyde and 3% glutaraldehyde in cacodylate buffer, a fixative which has been developed for plastic embedding and ultra-thin sections. The tissue is allowed to fix for 12 h at room temperature before being washed for a total of 4 h with two changes of tap water. Dehydration is carried out for 1 h in 70% ethanol

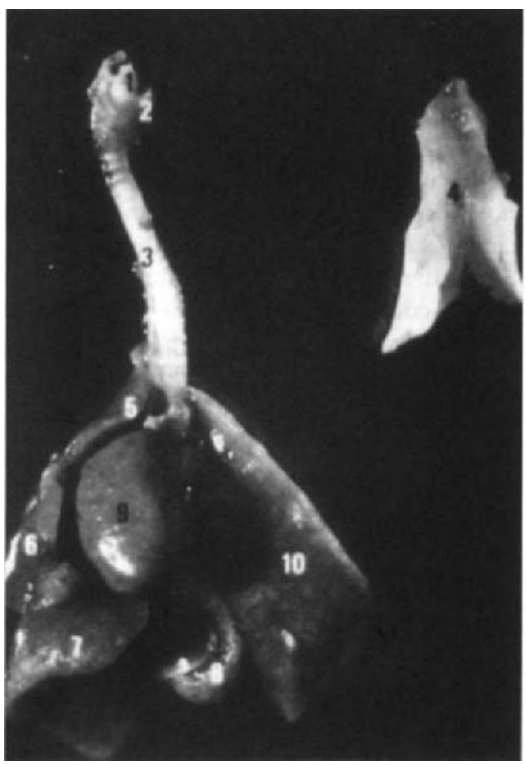


Figure 15. Rodent lung and heart: 1, epiglottis; 2, thyroid cartilage; 3, trachea; 4, thymus; 5–8, cranial lobe, medial lobe, caudal lobe, accessory lobe; 9, heart; 10, left lung (Olds and Olds, 1979).

and for another hour in absolute ethanol using constant agitation. Subsequent embedding in acrylic resin and further processing should be done in collaboration with a pathologist.

The lung requires special attention, as it should be inflated with 1 ml of fixative via a 26-gauge needle inserted into the trachea prior to removal. The fluid should be retained in the lung by closing the trachea with a suture. Care has to be taken when the entire heart–lung package (Fig. 15) is removed, because the lungs are attached to the dorsal pleura at their cranial part via membranes. Lack of care during removal may thus result in tissue disruption and leakage of the fixative, and interpretation of lung lesions is complicated by atelectasis. Careful perfusion of the kidneys or liver via the renal or portal vein after incision of the vena cava inferior may, in some cases, increase the quality of histological examination. Tissue is cut into appropriately sized pieces, placed in plastic forms (Crymold, Miles Inc., Elkhart, IN), filled with OCT compound (Miles), and snap frozen in liquid nitrogen and stored at –70°C until further processing. For more details on this procedure, see the chapter by Ehlers and Seitzer (p. 365ff).

In order to obtain reproducible data, at least three slides from different levels of each organ should be examined by an investigator blinded to the experimental conditions. Lesions may be quantified on a number/area or area/area basis (e.g. 0.25 cm^2). Only slides prepared simultaneously and of identical thickness should be compared. Photography increases accuracy and should include calibration information (scale bars).

Investigating inflammatory responses in various compartments: collection of specimens

In addition to histological examination of infected tissues, inflammatory host responses may also be followed by detecting cellular and molecular changes in blood and serum, the peritoneal cavity or the alveolar space. Since there are significant strain-, age- and method-dependent differences in the composition of inflammatory exudates of mice, normal values should always be established for each laboratory. The following data are presented only to give a rough idea of what can be expected. The values given herein represent data from adult mice.

Blood. For the collection of blood (maximum 1 ml per mouse), mice are sacrificed by CO_2 inhalation and bled within 1–2 min via the heart or brachial vessels. The heart is exposed via an incision in the ventral thoracic area and blood aspirated directly from the right ventricle using a 1- to 2-ml syringe and a 20- to 25-gauge 1-inch needle. Since murine blood clots very fast, it may be helpful to preinject 25 U of heparin in 0.5 ml PBS intraperitoneally 30 min before collection.

Retro-orbital bleeding. Retro-orbital bleeding is a method of obtaining serial blood samples from mice. It is performed with a Pasteur pipette or a capillary tube, and usually yields between 50 and 100 µl of blood. Anaesthesia (see above) should always be used. The Pasteur pipette or capillary tube is heparinized by dipping the pipette or tube into heparin (1000 IE ml^{-1}) and letting the heparin rise into the tube via capillary action.

After blowing out the heparin, the pipette or tube is inserted between the nasal epicanthus and the eye and pushed slowly forward with rotation around the longitudinal axis. It is important not to use too much force since the pipette or tube may break. Any injury to the eye must be strictly avoided. Puncture of the retro-orbital venous plexus is achieved when blood starts to flow, sometimes with astonishing speed. After obtaining the blood sample by capillary action, the pipette or tube is removed. Usually, blood stops to flow on removal of the pipette. The animal is allowed to recover and can be bled again on the following day, but the contralateral eye should be used.

Blood can be analysed using a blood film slide spread. It is allowed to dry in air and subsequently stained with May–Grünwald–Giemsa or Diff Quick stain. It should be mentioned that the normal nuclear pattern of neutrophils in the mouse includes ring forms and that the cytoplasmic granulation is very fine. The nucleus of eosinophils is usually coiled and contains small acidophilic granules. The lymphocyte is the prevalent cell type in mouse blood. The nucleus of the monocyte is ameboid in shape, with stranded chromatin. Small vacuoles and a few acidophilic granules are usually present in the grey-blue cytoplasm (Table 6).

Serum. Serum is obtained after blood has been allowed to clot for 1 h at room temperature and subsequently has been centrifuged at 3000*g* to 4000*g*. For the determination of cytokines in serum, it is advisable to centrifuge heparinized blood immediately after collection at 4°C. Microcapillary tubes containing a separating gel (Microtainers, Becton Dickinson & Co., Rutherford, NY) may facilitate serum separation and increase the yield. For composition of serum see Table 7.

The inflammatory response to bacteria can be studied in exudates of the lung or the peritoneal cavity. However, there is significant strain dependency in the intensity of inflammation in response to the same

Table 6. Cellular composition of murine blood

Red cells	8×10^6 to $10 \times 10^6 \mu l^{-1}$
Leukocytes	3×10^3 to $22 \times 10^3 \mu l^{-1}$ (strain and source dependent)
Monocytes	1–14%
Neutrophils	7–28% (strain dependent)
Lymphocytes	65–80%! (strain dependent)
Eosinophils	1–4%
Basophils	< 1%
B cells	20% of lymphocytes
T cells	80% of lymphocytes
$CD4^+$ T cells	52% of lymphocytes
$CD8^+$ T cells	26% of lymphocytes
$CD4^-CD8^-$ T cells	0.5–2% of lymphocytes
NK cells	6–11% of mononuclear cells

NK, natural killer.

Table 7. Composition of murine serum

Total protein	4–5 g per 100 ml
Albumin	61–70%
α-Globulin	10–20%
β-Globulin	12–20%
γ-Globulin	5–10%
Total immunoglobulin	2.3–6.6 mg ml^{-1}
IgM	0.06–0.17 mg ml^{-1}
IgA	0.08–1.55 mg ml^{-1}
IgG_1	0.14–3.2 mg ml^{-1}
IgG_{2a}	0.89–3.7 mg ml^{-1}
IgG_{2b}	0.38–1.9 mg ml^{-1}
IgG_3	0.06–0.14 mg ml^{-1}

stimulus. Balb/c, A/J and DBA mice usually show low inflammatory responses, while C57BL/6 and C3H/HeJ are high responders.

Peritoneal cavity. Resident and elicited cells of the peritoneal cavity can be obtained by peritoneal lavage (Plate 4). After cleaning the abdomen with 70% ethanol, the skin is incised and the peritoneal cavity prepared by dissecting the skin cranially and caudally. Peritoneal cells, including resident macrophages, can then be harvested by lavaging the peritoneal cavity with 5–8 ml cold PBS supplemented with 10 U ml^{-1} heparin. A 19-gauge needle or a pipette is inserted in the umbilical region. The fluid should be withdrawn carefully in order to prevent aspiration of abdominal structures, which may result in obstruction, intestinal damage and bacterial contamination of the lavage fluid. Recovery of 70–90% of the injected fluid should be the goal. The cells are washed in PBS by centrifugation at 450*g* for 10 min (Table 8).

Table 8. Murine resident peritoneal cells as obtained by peritoneal lavage

Total cells	2×10^6 to 4×10^6
Macrophages	50–70%
Lymphocytes	25–50%
B cells	N/A
T cells:	
$CD4^+$	74% of T cells
$CD8^+$	18% of T cells
$CD4^-CD8^-$	8% of T cells
Neutrophils	2%
Eosinophils	< 1%
Mast cells	< 1%
NK cells	< 0.5%

NK, natural killer.
N/A, not available.

Alveolar space. Lavage of the non-inflamed alveolar space is an excellent source for almost pure preparations of alveolar macrophages. Bronchoalveolar lavage obtained from mice suffering from pneumonia is helpful in characterizing the inflammatory response. This is easily performed using a 26-gauge Teflon catheter (e.g. Abbocath). After sacrifice, the mouse is placed on its back and fixed on the plate. After cleaning the skin with 70% ethanol, the skin is incised and removed from the mid-abdomen up to the chin. A laparotomy and thoracotomy is performed with sterile instruments. The trachea is prepared by carefully removing the thyroid gland and the muscles covering the trachea using atraumatic surgical techniques. Then, the scissors are inserted into the jugular fossa and the sternum is cut lengthwise towards the abdomen to expose the entire mediastinum. A small tunnel is prepared under the trachea by inserting closed scissors and pushing them forward using the forceps as a resistor. A suture is prepared by pulling an appropriate thread through the tunnel. Then, the Teflon catheter is inserted into the trachea and the hypodermic needle is removed. It is important to insert the catheter at the most cranial point possible so that, if the trachea is perforated accidentally, another, more caudal, insertion is still possible. The catheter is secured by fastening the previously laid suture. The lungs are then lavaged with a maximum of 1 ml sterile physiological saline containing 0.05 M ethylene diaminetetraacetic acid (EDTA). The lavage should be repeated up to 20 times, yielding a total of 20 ml lavage fluid. It is important not to apply too much pressure when injecting the fluid or too much suction when aspirating it, otherwise large blood vessels may rupture and the lavage becomes 'contaminated' with cells from the blood. A non-infected mouse yields about 1×10^5 to 5×10^5 alveolar macrophages in total. Plate 5 shows the typical cellular composition of a bronchoalveolar lavage as assessed by cytospin preparation and Diff Quick stain (Baxter Scientific, McGav Park, IL, USA). The presence of red blood cells indicates that some rupture of capillaries is unavoidable, but there should be no more than 5% of granulocytes in the lavage obtained from a healthy mouse.

◆◆◆◆◆◆ IDENTIFYING SPECIFIC HOST RESPONSES *IN VIVO*

B-cell Response

The B-cell response to bacterial infections can be studied by determining the amount of specific antibodies in serum and/or mucosal secretions such as intestinal and bronchoalveolar fluid (see above) by the use of enzyme-linked immunosorbent assays (ELISAs) (see the chapter by Fujihashi *et al.* (p. 257ff). The specificity of antibody responses can easily be determined by Western blot analysis. Because in the mouse there is a close relationship between the IgG subclass and T-cell help by T helper 1 and 2 (Th1 and Th2) cells (IgG_{2a}/Th1 and IgG_1/Th2), determining subclass

of specific antibodies may be the first hint of the dominant ongoing T-cell response. The frequency of antigen-specific B cells in blood, lymph nodes, spleen, lung, Peyer's patches or cells of the BALT can be determined using the ELISPOT assay (see the chapter by Fujihashi *et al.*, p. 257).

Collecting faeces and intestinal fluid for detection of secretory IgA

Detection of secretory IgA in stool specimens can be performed as described by Haneberg *et al.* (1994). During the day, 1–10 stool specimens are collected. The samples are then dried using a speed-vac concentrator. After determination of their weight, the stool specimens are rehydrated and homogenized in PBS containing protease inhibitors such as bestatin (154 nM apotinin, 10 μM leupeptin, 200 μM 4-(2-aminoethyl)benzol-sulfonylfluoride-hydrochloride (AEBSF; molecular weight 239.5), 6 μM bestatin) and dry milk powder. The homogenate is centrifuged and the supernatants are collected. This extraction procedure should be repeated once, and the final extracts are then snap frozen in liquid nitrogen and stored at –70°C until analysis.

An alternative method involves the use of absorbant wicks (Polyfiltronics Inc., Rockland, MA, USA) to collect intestinal fluid (Haneberg *et al.*, 1994). After removing the wicks from the intestine, they are centrifuged for 5 min at 10 000*g* in 0.5-ml Eppendorf tubes, which are pierced at the bottom and placed in a large Eppendorf tube (1.5 ml) without a lid. After centrifugation the samples can be collected at the bottom of the large Eppendorf tube. Alternatively, protease inhibitors (see above) should be added in order to avoid degradation of IgA antibodies by proteases within the secretion fluid. Faecal samples can be dissolved in 5% powdered milk using a vortexer. The fluids should be aliquoted, snap frozen in liquid nitrogen and stored at –70°C, as secretory IgA antibody may form aggregates that make subsequent studies nearly impossible.

Gut washes

The small intestine is flushed with 2 ml PBS containing protease inhibitors. The fluid is centrifuged for 5 min at 1000*g* to remove debris and bacteria. The remainder of the procedure is as described above.

T-cell Response

T-cell-mediated immunity is expressed as cytotoxicity (see the chapter by Bush and Pamer, p. 237ff) or the release of cytokines (see the chapter by Fujihashi *et al.*, p. 257ff). The characteristic *in vivo* manifestation of the latter is delayed-type hypersensitivity and granuloma formation. The reaction involves a specifically directed infiltration of mononuclear cells into an area where antigen is localized. The hypersensitivity is referred to as 'delayed' as it takes some 16–24 h to become fully apparent. An essential characteristic is that the reaction is independent of serum antibodies. Great care should be taken to differentiate the DTH reaction from the

Arthus reaction. Arthus reactions, however, reach their maximum 4-6 h after application of the antigen, so that these time points should always be included in monitoring the skin response to intradermally injected antigen, especially when Gram-negative bacteria are used. Histologically, DTH is characterized by accumulation of mononuclear cells around small veins. Later, mononuclear cells may be seen throughout the area of the reaction, with extensive infiltration of the dermis. Polymorphonuclear cells constitute fewer than one-third of the cells at any time, and usually very few are present at 24 h or later unless the reaction is severe enough to cause necrosis, which is not typical in the mouse. Some of the swelling may be due to the packing of the tissues with these cells, although oedema and hyperaemia also play a role. The $CD4^+$ and $CD8^+$ subsets of T cells in the early perivascular areas are in the same ratio as in peripheral blood, but the cells in the late diffuse infiltrates in the dermis are predominantly $CD4^+$ T cells. In fact, the *in vivo* depletion of the latter (see p. 352) 24 h before challenge will ablate the reaction.

The tuberculin reaction, as discovered by Koch in 1891, is the prototype of these mechanisms. However, it has certain disadvantages, the main of which are that work with *M. tuberculosis* is hazardous and that the complex cell-wall composition of the tubercle bacillus complicates the study of mechanisms involved in sensitization.

A deeper insight into the mechanisms underlying DTH and cellular immunity developed after the classic work by Chase and Landsteiner (see Table 1). During the 1960s, the importance of cell-mediated immune responses in defence against bacterial infections was emphasized by Mackaness, mainly by use of the murine listeriosis model. Therefore, T-cell responses may well be studied *in vivo* by analysing protective mechanisms against *Listeria monocytogenes* and *Listeria*-induced T-cell-mediated inflammation (Mielke *et al.*, 1997).

Delayed hypersensitivity is a complex *in vivo* reaction, which cannot be duplicated *in vitro*, involving non-specific perivascular accumulation of primed T cells, activation of specific T cells by antigen presenting cells, production of cytokines, which subsequently induce the site-directed accumulation of monocytes mainly via cell derived chemokines and the induction of adhesion molecules on endothelial cells. The only *in vitro* correlate available reflecting part of the reaction is the production of IFN-γ by specific $CD4^+$ T cells (see the chapter by Fujihashi *et al.*, p. 257ff). In the mouse, two types of DTH have to be differentiated: the tuberculin type and Jones–Mote type. Tuberculin-type hypersensitivity, originally observed in tuberculous guinea-pigs, is the classical type of T cell-mediated inflammation, lasting 48 h even in mice. It is elicited in previously sensitized individuals by intradermal injection of protein antigens. Although it can also be elicited by heat-killed bacteria, the degree of non-specific swelling is generally higher when particulate antigen is used.

The term 'Jones–Mote reaction' originally referred to the delayed reappearance of a hypersensitivity reaction to serum proteins after the development and regression of an Arthus reaction, noted in humans by Jones and Mote in 1934. This term was extended to cover a transient form of delayed skin reaction to protein antigens occurring prior to antibody

production in experimental animals. While the Jones–Mote type of lymphocyte-mediated inflammation may be suppressed by CD11b-specific mAb, the classical type cannot. It is not clear whether both types of DTH are mediated by different subsets of $CD4^+$ T cells (Ignatius *et al.*, 1994).

The time course of the reaction, is a very unreliable criterion to differentiate between these types of hypersensitivity reactions, so that histology should always be performed demonstrating pronounced PMN accumulation in the Jones–Mote type reaction.

Acquired resistance is not influenced by simultaneous measurement of DTH in the same animal, but challenge of primed animals with a large dose of bacteria or highly virulent micro-organisms may impair the expression of DTH. DTH reactions, although in most cases demonstrated in the skin, may be elicited in various organs when particulate antigen is injected intravenously. Under these conditions it will present as focal accumulations of monocytes and lymphocytes in tissue, i.e. granulomas. Therefore, challenging immunized mice with heat-killed bacteria and quantifying inflammatory tissue lesions will be an alternative method for investigating $CD4^+$ T-cell-mediated inflammation *in vivo*. While granulomatous lesions are difficult to determine in lymphatic organs such as lymph nodes and spleen, quantification is easy in a parenchymal non-lymphoid organ like the liver. Reliable quantification is possible if more than 10^6 particles are injected intravenously and the liver is removed 48–72 h after injection. However, no sequential determinations within the same animal are possible using this read-out. Antigen complexed to insoluble particles, such as latex beads, will also produce granulomas when injected into immunized animals, and therefore will enable the investigator to characterize the host response to defined antigens (Brocke *et al.*, 1991). This may be important if non-degradable material is present in the bacterial preparation, as in mycobacteria, since under these conditions, T-cell-independent granulomas may occur. The great advantage of this method is easy histopathological analysis. The composition of granulomatous lesions may vary depending on the type of Th response (e.g. cercaria-induced granulomas (*Schistosoma mansoni*) may contain 50% eosinophils, 30% macrophages, 10–15% T cells, 5–10% other cells).

While $CD4^+$ T-cell-mediated responses such as DTH and granuloma formation can easily be detected, T-cell-mediated cytotoxicity is much more difficult to demonstrate. In fact, there is no model of bacterial infections in which the cytotoxic action of T cells has been shown directly using fresh, non-restimulated T cells *ex vivo*. Consequently, the effect of $CD8^+$ T-cell-subset-depletion may be the only way to demonstrate their action *in vivo*.

Preparation of soluble antigen and antigen-coated latex beads

Soluble antigen from bacteria may be obtained by ultrasonication of heat-killed bacteria. Bacteria (1 g wet wt) is suspended in 10 ml PBS. This suspension is then sonicated 5–10 times for 30 s to 1 min each, on using, for example, a Branson Sonic Power sonifier placed in a safety cabinet. The sonicated suspension is centrifuged at 40 000*g* for 1 h and the soluble fraction is filter sterilized using a 0.22-μm pore filter. The protein concen-

tration of this stock solution has to be determined and aliquots are stored frozen at –70°C.

An alternative method of obtaining soluble antigen, especially when secreted metabolic antigens are required, is to inoculate protein- and antibiotic-free RPMI 1640 cell culture medium with 1×10^8 bacteria. The culture is constantly agitated using Cellspin bottles (Bioscience, Inc., Tecnomara, Chino, CA, USA) and then incubated in an atmosphere of 5% CO_2 in air for time periods depending on the type of bacterium (16 h to 7 days). Subsequently, the suspensions are centrifuged and filter sterilized. The filter-sterilized supernatant may be concentrated by ultrafiltration (amicon, millipore).

DTH is usually elicited by intradermal foot pad injection (see above) with 3–5 µg of soluble antigen in PBS in a volume of 50 µl. The injection site should be inspected after 6, 24 and 48 h. The thickness of each hind foot is measured with a dial-gauge calliper (Fig. 16). Specific foot-pad swelling is reported as (Right minus left foot-pad thickness in primed mice) – (Right minus left foot-pad thickness in mice that received vehicle only).

Coating of latex beads (3 µm diameter, Sigma, Germany) with soluble antigen can be achieved by washing the native beads in PBS, collecting them by centrifugation at 1200*g* for 10 min, resuspending them at a concentration of 10% vol./vol. in PBS containing the soluble bacterial antigen or an irrelevant protein such as ovalbumin at a concentration of 30 µg ml^{-1}. The beads are then co-incubated with the antigen for 1 h at room temperature, washed twice in PBS, and subsequently injected intravenously at a dose of 200 µl of a 10% vol./vol. suspension per mouse (Brocke *et al.*, 1991).

An alternative method to elicit T-cell-mediated monocyte accumulation in the liver is to use heat-killed bacteria injected intravenously at a dose of 2×10^8 per animal. This dose will result in a quantifiable density of granulomas per liver section (Mielke *et al.*, 1992).

Cytokine Response

Bacterial infections usually induce strong cytokine responses that can be determined both systemically (in serum) and locally at both the organ and

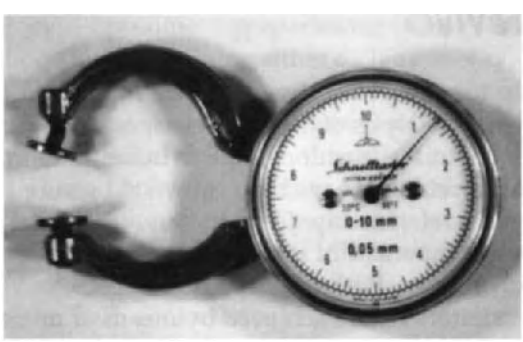


Figure 16. Dial-gauge caliper for measuring foot-pad thickness.

the cellular level *in situ*. The cytokine response to bacteria starts almost immediately after infection, as macrophages begin to transcribe message from a number of early response genes and cytokines such as IL-1, IL-6, IL-10, IL-12, macrophage chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, granulocyte, macrophage and granulocyte–macrophage colony-stimulating factors (G-CSF, M-CSF, GM-CSF) and tumour necrosis factor α (TNFα), which in turn induce the production of IFN-γ by natural killer (NK) cells.

Global cytokine responses may be monitored by measuring cytokine levels in serum (see the chapter by Fujihashi *et al.*, p. 257ff). Therefore, at the organ level, cytokines may best be determined by semiquantitative polymerase chain reaction (PCR) or RNase protection assays (see the chapter by Ehlers and Seitzer, p. 350ff). Another relatively reliable method of obtaining information about infection-induced cytokines is to measure spontaneous and antigen-induced cytokine production in spleen cell cultures (see the chapter by Fujihashi *et al.*, p. 257ff) obtained at various time points after infection. The production of cytokines at the cellular level may be demonstrated by immunohistology or by *in situ* hybridization (see the chapter by Ehlers and Seitzer, p. 350ff).

For the determination of cytokines in organ homogenates, tissues should be weighed and suspended in 10 times their weight of cold RPMI 1640 containing 0.2% BSA, 0.02% sodium azide and 1% wt/vol. (cholamidopropyl)-dimethylammonio)-1-propane sulfate (CHAPS; Wako Pure Chemical Co., Osaka 541, Japan). In this solution, tissue is homogenized on ice and the homogenate is centrifuged at 1000*g* for 10 min at 4°C. The supernatant may be filter sterilized using low protein binding filters and stored in aliquots at –70°C until determination of cytokine levels using specific ELISAs (see the chapter by Fujihashi *et al.*, p. 257ff).

For mRNA analysis, organs should be excised, weighed and immediately homogenized in guanidine isothiocyanate (4 M guanidium isothiocyanate, 0.5% *N*-lauroylsarcosine, 25 mM sodium citrate, 100 mM 2-mercaptoethanol) and frozen at –70°C for future use in preparing mRNA (see the chapter by Fujihashi *et al.*, p. 257ff).

◆◆◆◆◆◆ MANIPULATING THE IMMUNE RESPONSE *IN VIVO*

Up to now descriptive methods have been discussed. However, phenotypical analysis of the various aspects of the host response does not tell us all about the functional role in inflammation and defence. In fact, temporal correlations have been shown to be unreliable in terms of elucidating a causal relationship. Only the loss of a certain function after depletion of one component of the immune system allows some causal inference. In this respect, both the depletion of cells and the neutralization of soluble mediators can be achieved by means of monoclonal antibodies or specific heterologous sera (see Table 11). This approach competes with the use of gene knock-out (GKO) mice (see the chapter by Mossmann *et al.*, p. 109),

but has certain advantages, especially in models in which the period of observation is short (up to 4 weeks). In contrast to GKO mice, the mAb-mediated depletion of certain cells or the neutralization of cytokines has no long-lasting effects that may favour compensatory mechanisms such as the increase in NK-cell activity in T-cell-deficient mice, or may even result in anatomical differences such as a lack of Peyer's patches (PP) in GKO mice deficient in TNF-RI. Therefore, whenever a transient, temporally defined immunomodulation is of interest, studies using antibodies for depletion or neutralization may be of advantage. In particular, in situations in which the role of a cell population either in the induction or the expression of immunity is to be explored, monoclonal antibodies are ideal tools. On the other hand, GKO mice are well suited to the demonstration of the compensatory potential of the still active cells and mechanisms.

Depleting PMNs, Macrophages and NK Cells

The SCID mouse is a well-established model for studying mechanisms of innate resistance, due to its inability to generate specific antigen receptors. In this context it may be interesting to abolish certain aspects of non-specific resistance by depleting PMNs, monocytes, resident macrophages or NK cells.

While the depletion of PMNs and NK cells can be achieved relatively easily by *in vivo* application of certain mAbs, the selective depletion of monocytes and/or resident macrophages is more difficult to achieve. To deplete mice of neutrophils, mAb RB6-8C5 (R. Coffman, DNAX Research Institute, Palo Alto, CA, USA) is administered intraperitoneally in a dose of 0.25 mg, 1 day prior to initiating infection. It has been shown that treating mice in this way renders them severely neutropenic for at least 3 days. Control mice should receive normal rat IgG (Sigma Chemical Co., St Louis, MO, USA).

The depletion of NK cells can be achieved by intraperitoneal application of 400µg rabbit anti-asialo-GM1 antibody (Wako Pure Chemical Co.) in 0.5 ml pyrogen-free PBS 2 days before challenge with antigen. Normal rabbit globulin should be used as a control. A single injection results in a marked reduction in splenic NK activity for at least 7 days.

Recently, a method for selectively depleting animals of resident macrophages by liposome preparations has been described. Multilamellar liposomes containing either dichloromethylene diphosphonate (Cl_2MDP; Boehringer Mannheim GmbH, Mannheim, Germany) or PBS may be prepared as described by van Rooijen and Sanders (1994). Briefly, 75 mg phosphatidylcholine (Lipoid GmbH, Ludwigshafen, Germany) and 11 mg cholesterol (Sigma Chemical Co., St Louis, MO, USA) are dissolved in chloroform, evaporated by rotation under vacuum at 37°C, dispersed by mixing with 10 ml PBS containing 2.5 g Cl_2MDP for 10 min, left at room temperature for 2 h, sonicated for 3 min at room temperature in a water bath sonicator, and left at room temperature for 2 h. The resulting liposomes containing Cl_2MDP (L-Cl_2MDP) are washed twice in PBS by centrifugation at 100 000*g* for 30 min to remove non-

encapsulated Cl_2MDP. PBS-containing liposomes (L-PBS) are prepared by the same procedure, except that the phosphatidylcholine–cholesterol mixture is dispersed in 10 ml PBS without Cl_2MDP. The L-Cl_2MDP and L-PBS are each suspended in 4 ml PBS, and 0.2 ml of either preparation is injected intravenously per mouse.

Depleting T-cell Subsets

An efficient method of elucidating the role of T-cell subsets *in vivo* is to utilize their selective depletion (Waldmann, 1989). However, antibodies induced against the injected monoclonal antibodies may limit their repeated use in long-term experiments. The only exception is the depletion of $CD4^+$ T cells by mAb GK 1.5 (ATCC TIB 207), for which it has been shown that no such antibodies will be produced. $CD8^+$ T cells may be depleted by IgG_{2b} antibodies from the rat hybridoma 2.43 (ATCC TIB 210), and for Thy 1.2^+ cells rat IgG_{2b} antibodies from clone 30H12 (ATCC TIB 107) can be used. Intraperitoneal injection of 400 µg of the mAbs per mouse will result in a profound depletion lasting about 7 days.

In order to prevent non-specific effects on the macrophage system by cell destruction, and to guarantee efficacy, mAbs should be injected 2 days before the start of the experiment. The efficacy of depletion has to be verified by fluorescence-activated cell sorting (FACS) analysis of splenocytes or Peyer's patches (see the chapter by Scheffold and Radbruch, p. 23ff) (Tables 9 and 10), at least at the end of the experiment. In order to prevent failure of cell detection by blockage of surface markers due to the antibodies given *in vivo*, a sandwich method should be used to label the cells.

Table 9. Cellular composition of murine spleen

Total number of cells	0.5×10^8 to 2×10^8 per spleen
B cells	65% of lymphocytes
T cells	35% of lymphocytes
$CD4^+$ T cells	20% (65% of T cells)
$CD8^+$ T cells	15% (32% of T cells)
$CD4^-CD8^-$ T cells	0.5–2% of lymphocytes (3% of T cells)
Monocytes	10% of total cells
NK cells	5% of total cells

NK, natural killer.

Table 10. Cellular composition of murine Peyer's patches

T cells:	20%
$CD4^+$	12%
$CD8^+$	8%
$CD4^-CD8^-$	50% of lymphocytes in intestine
B cells	80%
IgG^+ B cells	70%
Phagocytes	3%

Neutralization of Cytokines

Cytokines can be neutralized *in vivo* by intraperitoneal administration of mAbs. The neutralizing capacity of the antibodies should be determined in order to verify the neutralizing effect, since the amount of antibody required varies significantly, ranging from 125 µg to 4 mg per mouse. In variation of the procedure used for cell depletion, neutralizing antibodies may be administered up to 4 h before infection or immunization. Table 11 provides a list of available neutralizing antibodies. Equal amounts of normal rat or rabbit immunoglobulin of the same isotype must be injected as a control.

Table 11. Cross-reactivity of human and murine cytokines and sources for neutralizing antibodies

	Man	Mouse	*In vivo* neutralizing antibody
IL-1	++	+	Genzyme
IL-2	++	+	Pharmingen (S4B6)
IL-3	++	–	Pharmingen (MP2-8F8)
IL-4	++	–	Genzyme, pharmingen (BVD4-1D11; 11B11)
IL-5	++	+?	Genzyme
IL-6	++	+	Pharmingen (MP5-20F3)
IL-7	++	++	Genzyme
IL-8	++	+	
IL-9			
IL-10	++	++	Genzyme, pharmingen (JES5-2A5, JES5-16E3)
IL-11	++	++	
IL-12	–		Genzyme, pharmingen (C17.8)
IL-13			
IL-14			
IL-15	++	++	
IL-16			
TNFα	++	+	Genzyme
TNFβ	++	+	
IFN-α	++	+	
IFN-β			
IFN-γ	++	–	Genzyme
MIP-1α	++	++	
MIP-1β	++	+	
MIP-2			
MCP			
RANTES			
G-CSF			
M-CSF	++	+	
GM-CSF	++	–	
TGF-β	++	+	Genzyme

The effects of administration of cytokines *in vivo* is even more dependent on dose and route of application. Some cytokines are very potent immunomodulators and act in minute concentrations, while other cytokines need to be present in high concentrations to promote immunomodulatory effects *in vivo*. Furthermore, co-administration of an infective agent may dramatically increase the effects of a cytokine. Cytokines may be administered intravenously or intraperitoneally, but usually act in a more protracted manner after subcutaneous injection. Compared to the value of *in vivo* neutralization, the usefulness of recombinant cytokines is much lower due to problems of pharmacokinetics, distribution and secondarily induced cytokines.

All reagents used *in vivo* should be tested for endotoxin contamination. Best suited for this purpose is the Limulus amoebocyte lysate assay (Associates of Cape Cod, Woods Hole, MA, USA; Bio Whittaker, Walkersville, MD, USA; Sigma), which detects amounts as low as 10 pg ml^{-1}. The goal is to inject no more than 10 pg LPS per dose.

◆◆◆◆◆ METHODS FOR ASSESSING IMMUNOLOGICAL MEMORY

Infectious processes may be divided into (a) an early phase, in which mechanisms of innate resistance are expressed and immunity is induced, and (b) an immune effector phase, which is rapidly switched off. Finally, a state of long-term immunological memory develops. If memory reflects the extent of previous lymphocyte proliferation that is maintained, the optimal method of measuring this may be to determine the frequency of specific T and B cells by limiting dilution analysis by using the relevant effector mechanism (e.g. cytokine or antibody secretion) for read-out (see the chapter by Fujihashi *et al.*, p. 257ff). If memory represents a change in the physiological status of, for example, IL-producing or cytotoxic T cells, rather than just an increase in numbers, differences in IL patterns and cytotoxic activity should be studied (see the chapter by Bush and Pamer, p. 237ff).

Protection against secondary challenge is chosen as the most important measure of memory *in vivo*. Kinetics of a challenge infection should be monitored in naive versus primed or adoptively immunized animals. However, because of the complexities of the latter parameters, these *in vivo* read-outs are sometimes difficult to interpret. For example, it may be particularly troublesome to evaluate the roles of B cell, Th or cytotoxic T-cell memory. Under these conditions, depletion studies (see above) are most helpful. In any case, to get a complete picture, both high- and low-dose challenges should be analysed in animals immunized with both high and low doses of bacteria.

In contrast to cytokine production by T cells, there is at present no *ex vivo* assay available for measuring directly the cytolytic activity of *in vivo* primed cytotoxic memory T cells with specificity for bacteria, making acquired immunity to secondary challenge the only reliable read-out.

Adoptive Transfer of Immunity

While studies based on the depletion of a certain immunologically important component will reveal whether it is *necessary*, the adoptive transfer of a phenomenon by serum or cells will reveal whether they are *sufficient*. It has to be kept in mind, however, that adoptive transfer studies correlate more with mechanisms expressed during secondary challenge than with those active during the eradication of a primary infection. The ability to transfer protection adoptively by means of 200–400 µl hyperimmune serum points to a role of antibodies in immunity to a secondary challenge.

Adoptive transfer of specific T cells may demonstrate their functional potency in mediating immunity when given before challenge. However, the interval after infection in which adoptive transfer of cell-mediated immunity is possible may be quite short, since it is often restricted to the immune effector phase, i.e. just before most activated cells undergo apoptosis and immunological memory develops. Cells able to transfer immunity may be enriched in the peritoneal cavity 3 days after the intraperitoneal injection of a sterile inflammatory irritant such as 10% proteose pepton in PBS. Peritoneal exudate T cells (PETLs) may then be obtained by peritoneal lavage (see above) and enriched by using methods (see the chapter by Czuprynski and Brown, p. 189) based on the adherence of macrophages and B cells to plastic surfaces. Usually 5×10^6 PETLs suffice to transfer a certain degree of immunity. Another method of transferring cellular immunity is based on spleen equivalents. The easiest way to do this is to disperse the cells of a spleen of a donor mouse (see the chapter by Czuprynski and Brown, p. 189) and to inject up to 1×10^8 cells intravenously in 0.3 ml PBS into the recipient. Adoptively transferred activated macrophages are usually unable to confer protection. However, in infections with intracellular bacteria, viable bacteria may be transferred with the cell suspension if macrophages are not depleted.

In order to track lymphocytes in tissues after adoptive transfer, cells can be labelled with fluorescent dyes such as PKH2-GL (Dianova, Hamburg, Germany). After incubation of cells with this compound the cells should be washed and thereafter injected intravenously into the animal. PKH2-GL allows tracing of cells in organs by FACScan or fluorescence microscopy for up to 4 days. Alternatively, cells can be labelled with ^{51}Cr and traced *in vivo* with a γ-counter (Hamann *et al.*, 1994).

◆◆◆◆◆ STATISTICAL ANALYSIS

Introductory Remarks

Despite all efforts to standardize experiments, *in vivo* results depend on such a tremendous number of influencing factors (intangible variance) that statistical analysis is of utmost importance. In addition, results have to be reproduced in independent laboratories before they can be taken as valid. Due to significant differences in basic parameters between various

mouse strains, observations made on one strain are 'true' for that strain only. General statements have to be proven in at least three different strains of mice.

General Considerations

Three statisticians and three epidemiologists are travelling by train to a conference. The statisticians ask the epidemiologists whether they have bought tickets. They have. 'Fools!' say the statisticians, 'we've bought only one between us!' When the ticket inspector appears, the statisticians hide together in the toilet. The inspector knocks and they pass the ticket under the door. He clips the ticket and slides it back under the door to the statisticians. The epidemiologists are very impressed, and resolve to adopt this technique themselves. On the return they purchase one ticket between them, and share the journey with the statisticians, who again ask whether they've all bought tickets. 'No', they reply, 'we've bought one to share.' 'Fools!', say the statisticians, 'we've not bought any.' 'But what will you do when the inspector comes?' 'You'll see.' This time when the inspector appears, the epidemiologists hide together in the toilet. The statisticians walk up to door and knock on it. The epidemiologists slide their ticket under the door, and the statisticians take it and use it as before, leaving the epidemiologists to be caught by the inspector.

This joke illustrates a major problem of statistics. With the advent of sophisticated statistical computer software, almost every statistical test is possible and easy to perform once the data have been entered into the database. It is merely a matter of pressing a button and one can have almost every possible evaluation within seconds. This, in turn, leads to a great temptation to do exactly that, and then go with the test which gives the best results, i.e. usually the lowest p value. On the other hand, indiscriminate use of statistical methods prevents the careful thinking about possible confounding, information and sample distortion bias. Thus, when it comes to statistics, the best advice is to talk to a statistician *before* an experiment is conducted, i.e. in the planning stage of a project. This ensures that one has the correct sample size, the proper means of analysing the data and the reassurance that, whatever the final results may be, one can rely upon them. Otherwise, one could be left without a ticket when the inspector (or, in the case of scientific publication, the reviewer) shows up.

This section does not give an in-depth description of statistical procedures. Many good books are devoted to this matter. Instead, brief guidelines are provided about how to employ the most common descriptive and analytical statistical methods used in the context of *in vivo* analysis of infectious processes. Also, a short paragraph is given on how to show data properly in the form of graphs and tables. Here, we rely mostly on the book by Kramer (1988), which we consider to be one of the best books on the subject of statistics because it gives sufficient detail to compute the most important tests 'by hand', yet is written in a style which makes it easy for the reader to comprehend fully what exactly he or she is doing.

Descriptive Statistics

For all practical purposes, statistics can be divided into two major groups: descriptive statistics and analytical statistics. Each is as important as the other. *Descriptive statistics* deal, as the name implies, with the description of a population or of a sample taken from a population. For a given variable, it is highly unlikely that all members of a particular population show the same value. Rather, there will be a range of different values with a rate or mean sometimes differing considerably from that of the entire population. Thus, repeated and large samples from the same population are likely to give rates or sample means very close to the population value and to each other. Sampling variation is therefore inversely related to the sample size. The main purpose of descriptive statistics is to condense and summarize individual data from a sample drawn from a particular population.

There are two different kinds of variable: continuous and categorial. *Continuous variables* are expressed as integers, fractions or decimals with equal distances between successive intervals. Most parameters measured in immunological experiments fall within this category: bacteria measured as load (cfu per organ), body weight, foot-pat swelling (DTH), antibody or cytokine levels, quantifiable inflammatory host responses (e.g. the number of inflammatory cells in peritoneal exudates), LD_{50} values, and so on. *Categorial variables* are those in which the data are placed into one of two (dichotomous) or more (polychotomous) discrete (i.e. non-overlapping) categories (e.g. alive versus dead in survival analysis). To describe a population or data derived therefrom, in the case of continuous variables one can use a *frequency distribution*. Shown in graphical form, this results in a histogram. The other most commonly used measurement summarizing continuous variables is the *mean* or average. If there is a symmetrical distribution of the data (e.g. a bell-shaped curve or histogram), the mean is the ideal measure. However, if there is more than one peak in the histogram or if the distribution of the data is characterized by some skewness (asymmetry), the mean is of no use. In such cases other measures like the median or mode should be used. In the case of asymmetrically distributed data, the *median* (also referred to as the 50th percentile) is indicated. This is the value for which 50% of the group have lower and 50% have higher values. In the case of more than one peak in the distribution of the data, the *mode* (the most common single value) is the way to go. In immunology, group sizes and sample numbers of animal experiments tend to be small. Hence, it is sometimes impossible to tell whether the sample distribution follows a bell-shaped curve. In such cases, the median should be used.

The spread of a sample distribution is described with one of three measures: the range (the interval between the lowest and the highest value in the distribution), the percentile range, or the standard deviation. Again, the most commonly used measure is the *standard deviation* (SD) having the same limitations as outlined above for its counterpart, the mean. The standard deviation can also be expressed as a percentage of the mean value, i.e. as the *coefficient of variation* (CV). The advantage here is that the value

of the CV is independent of the units of measurement, thus allowing meaningful comparisons to be made. Absolutely robust against any outliers is the *range*. Somewhere in between those two are the *percentile ranges* (e.g. the quartiles, the range including all values between the 25th and the 75th percentile).

There is another sample statistic that occurs quite often in scientific literature to describe the distribution of data, but which is almost always used inappropriately. This is the *standard error of the mean* (SEM). Quoting Kramer (1988):

> Because the SEM decreases with increasing sample size, however, it is not a good descriptor of the spread of a frequency distribution, despite its popularity. A large sample with a high standard deviation (i.e. a wide spread) may have a small standard error. Since the SEM is always smaller than the standard deviation, it gives the impression that the spread of the data is less than it really is. Consequently, it may be favoured by authors who wish to minimize, rather than summarize, the variability of their data. The use of so-called error bars (defined by ± 1 SEM) above and below mean values displayed on a graph is a common example of this practice. The SEM is actually the standard deviation of a distribution of means obtained in repeated sampling from a source population. [The SEM] is important in making statistical inferences based on sample means. As a descriptor, however, it should be avoided.

In other words, just because the SEM is used virtually everywhere and makes 'nice and tight error bars' does not justify its use in graphs.

Among many possible distributions of sample data, the normal distribution is the most important. The distribution is described by just two parameters (mean and standard deviation), which makes it relatively easy to handle. Sometimes, if the data are not normally distributed, a transformation (e.g. the logarithms of the values instead of the values themselves) may result in a normal distribution of the now transformed data. There are some tests available to check whether or not a given set of data is indeed normally distributed. For practical purposes, however, it is simpler and faster to see whether the quotient of the median divided by the mean gives a value between 0.9 and 1.1. This indicates that there is no large difference between median and mean and an aggravating skewness in the distribution of the data is unlikely. The second check should be whether the coefficient of variation is less than equal to or 33%. This reveals whether another characteristic feature of a normal distribution is present, i.e. the fact that 67% of all values are within ±1 SD of the mean. If both conditions are present, one can be confident that the distribution of the values is a normal one. This is important when deciding which statistical test to use.

Testing a Hypothesis

Usually, one looks for an association between an exposure and a certain outcome and whether this association might have occurred by chance, i.e. whether the association is a true one. To do this, a *null hypothesis* (H_0) has

to be formulated, which (by convention) states that there is no association between exposure and outcome in the investigated population. This hypothesis is then tested by calculating the probability of obtaining, by chance alone, a degree of association between exposure and outcome at least as strong as that observed in the sample. This *probability* is the p value. If p is small enough (by convention, less than 0.05), the null hypothesis is considered to be unlikely and is thus rejected. Consequently, the *alternative hypothesis* (the actual research hypothesis) is considered to be likely enough that an association between exposure and outcome is probably not due to chance alone. Now, formulating the research hypothesis also implies that a statement is made as to how the exposure might affect the outcome, e.g. there will be an increase in TFNα levels in serum after injection of LPS. This is called a *directional hypothesis* (one-sided), and a directional (one-sided) test of H_0 is required. Often, however, there is no way of telling in advance whether an exposure will give rise to a particular outcome or whether the opposite will happen. This calls for a *non-directional* (two-sided) test of H_0. The p values given in tables or generated by computer programs apply to non-directional testing of H_0. Since the distribution of test statistics often has two tails, and since p is equal to the area under the curve in these two tails, it is called a two-sided p value derived from a two-sided (two-tailed) test. If the research hypothesis is directional and the outcome is indeed in the expected direction, a one-sided p value can be used, this being obtained simply by dividing the two-sided p value by 2. Both conditions, however, must be met before one can use a one-sided test. In uncertain cases, a two-tailed test should be used and a two-sided p value should be reported. The consequences of using a one-sided test when a two-sided one should have been employed instead can be quite dramatic. If the two-sided p value is 0.07, the corresponding one-sided p value will be 0.035. The former would be considered statistically non-significant, and the latter would suggest an acceptable risk of rejecting H_0.

If more than one question is to be answered in one experiment, usually more than two tests of significance are performed. However, this means that some significant exposure–outcome associations are likely to arise by chance. In turn, the null hypothesis would be rejected erroneously, resulting in a so-called *type I error*. To protect against this possibility, some statisticians advocate dividing the threshold α-level for rejecting the null hypothesis by the number of tests performed (the *Bonferroni correction*). Others argue, that this procedure may be overly conservative and tends to attribute true differences to chance because many outcomes are associated with one another, and therefore the probability of their joint occurrence is greater than the product of their individual probabilities. Hence it has been suggested that the number of tests performed should be reported along with the number achieving statistical significance. (For discussion of the *type II error* and *statistical power*, the reader is referred to more detailed sources (Kramer, 1988; Hays, 1994).)

Analytical Statistics

Statistical procedures within this category can be divided into several groups according to the characteristics of samples or populations to be tested. First, there are dependent and independent samples, and there are different tests for each condition. *Dependent samples* are usually derived from one individual in whom one variable was measured at least twice at different time points, e.g. cytokine levels in serum before and after infection. This implies that the size of the sample distribution is identical between the two parameters. On the other hand, *independent samples* are derived from individuals in whom a variable was measured only once, e.g. serum cytokine levels in infected and non-infected control animals. This means that the groups to be compared may be of different size.

Second, there are parametric and non-parametric tests. The parameters and descriptive statistics derived from *parametric tests* are used to detect differences between two outcomes and to draw statistical inferences based on these results. This in turn demands that all the source populations independent of each other do not violate the requirements of the *central limit theorem*. This theorem states that:

> If a population has a finite variance σ^2 and a finite mean μ, the distribution of sample means from samples of N independent observations approaches the form of a normal distribution with variance σ^2/N and mean μ as the sample size N increases. When N is very large, the sampling distribution of χ is approximately normal.

Nothing is said in this theorem about the form of the population distribution. Regardless of the population distribution, if the sample size N is large enough, the normal distribution is a good approximation to the sampling distribution of the mean. This in turn means that there is a very intimate connection between the size of a sample N and the extent to which a sampling distribution approaches the normal form.

On the other hand, all normally distributed samples meet the requirements of the central limit theorem. Thus, checking for normal distribution in the samples, as described above, is a simple and quick way to avoid trouble due to the use of an inappropriate statistical test, e.g. a parametric test such as the Student's *t*-test when a non-parametric test such as the Mann–Whitney *U*-test should have been used. As can be inferred from the above statement, there is basically one way to get data to fit a normal distribution or not to violate the central limit theorem. This consists of increasing the sample (population) size. In real life, however, this is not always possible. In this situation, which is commonly faced by immunologists, one has to switch to *non-parametric tests*. Non-parametric tests ignore the actual magnitude of the sample values and use ranks to determine statistical significance. In these tests, the sample values are ranked according to their magnitude, and from these ranks numbers are derived which are then used to calculate *p* values.

If categorial variables are considered, distributions (proportions) are compared rather than continuous values. One example could be the proportions of surviving and dead animals investigated in two different

groups, one receiving a specific treatment such as vaccination and the other receiving placebo. These data can be displayed in a 2×2 table. It is important to keep the null hypothesis in mind, i.e. that there is no association between exposure and outcome. To test this null hypothesis, expected frequencies that would correspond to the situation described in the null hypothesis are calculated. Taken together with the actual data, the observed frequencies, the chi-square (χ^2) test is then performed. This test uses observed and (calculated) expected frequencies in the four cells of a 2×2 table to compute a χ^2 value which then is used to decide whether the expected cell frequencies are indeed different from the observed frequencies. For mathematical reasons, the χ^2 test is always two-sided. For small sample sizes ($5 < n \leq 60$), the *Yate's correction* is the most widely used continuity correction. This results in smaller values for χ^2 and thus is more conservative. This reduced risk in erroneously rejecting the null hypothesis (type I error) is, of course, accompanied by an increased risk of not rejecting H_0 when in fact H_0 is untrue (type II error) and by a loss of statistical power. One way to circumvent this problem is to use the Fisher exact test, which must be used anyway if the expected frequency in one of the four cells of a 2×2 table is equal or less than 5. About 20 years ago, this would have required extensive calculations by hand, but now the performance of the Fisher exact test is not a problem anymore and should be done (according to some statisticians) regardless of the sample size.

So far tests have been suggested for cases in which one or more variables are categorial, i.e. in which one parameter observed in two distinct (categorial) groups is compared. For the case when both variables are continuous, a typical example being the dose dependency of cytokine responses to a particular stimulus such as LPS, the linear correlation should be determined, although it is possible to transform one continuous variable into a categorial one by defining non-overlapping groups (categories) and then to use the tests mentioned above. This, however, often results in a loss of data. Linear correlation measures the degree to which an increase in one of the variables is associated with a proportional change in the other variable. Displaying the variables graphically in the form of a scatter plot, one would find that, in the case of a perfect correlation, every point would fall onto a straight line. Since a few outliers can influence the correlation quite considerably, it is always advisable to plot the data and inspect the correlation visually before proceeding to further tests. If, for example, most of the data are located within a small area forming a diffuse cloud, but a few are located in the upper right-hand and lower left-hand sides of the plot, a test could reveal a statistically significant correlation when in fact there is none. Visual inspection of the scatter plot can reveal such clusters, and thus prevent reporting false-positive correlations.

The *Pearson correlation coefficient* (r) is the descriptive statistic most commonly used to describe a linear correlation. In analogy to the total variance of any continuous variable (which is the square of its standard deviation and a measure of the spread of the values around the mean), r^2 is a measure of the proportion of variance due to the linear correlation of one variable with the other. Thus r^2 is a good estimate for the amount of

linear correlation: the higher is r^2, the more likely is a linear relationship between the two variables. It is important to realize that the statistical significance of the correlation coefficient depends highly on the sample size. Thus, a large sample number requires only a small degree of correlation to achieve statistical significance, and a high degree of correlation might not be significant when the sample size is small.

No experiment is complete without reporting the results. Here also one should adhere to statistical conventions. Recently, the American Society for Microbiology gave some general guidelines in their information for contributors. One of the most common mistakes is the graphical display of non-normally distributed data using the mean and standard deviation. In this case the median and an appropriate percentile range should be used instead. As a matter of fact, the hints given above on how to check whether data are normally distributed or not can easily be used to examine whether the spread of a frequency distribution has been shown appropriately. It is quite quick and easy to estimate visually whether the standard deviation is more than a third of the mean (i.e. whether the coefficient of variation is greater than 33%). One possible way to circumvent such problems is to report values as the mean and the 95% confidence interval. This way of showing data is appropriate for normally distributed and non-normally distributed data.

Finally, we give a short example, the determination of the LD_{50} of a given bacterial strain, in order to illustrate how this important parameter is calculated. Consider that with 1.07×10^7 cfu 4 out of 10 mice (40%) died and with about 10-fold higher dose (1.15×10^8 cfu) 6 out of 10 mice (60%) succumbed to infection. The LD_{50} is then calculated according to the following equation:

$$\log LD_{50} = \log D_N + \left(\frac{50 - \%\ \text{Death at } D_N}{\%\ \text{Death at } D_V - \%\ \text{Death at } D_N} \times \log DF \right)$$

D_N is the dose at which the percentage of deaths is just below 50%, D_V is the dose at which the percentage of death is just above 50%, and DF = D_V/D_N. If 40% mortality occurred at a dose of 1.07×10^7 cfu and 60% mortality at a 10.75-fold higher dose, then

$$\log LD_{50} = \log (1.07 \times 10^7) + \left(\frac{50 - 40}{60 - 40} \times \log 10.75 \right)$$

$$= 7.0294 + \left(\frac{10}{20} \times 1.031 \right)$$

$$= 7.5449$$

All that is left to do now is to obtain the antilog of this number, resulting in our case in an LD_{50} of 3.51×10^7 cfu.

This method is, of course, applicable to the estimation of other endpoints, e.g. the dose of a pathogen that is needed to infect a given per-

centage of animals, the number of cells in a tissue culture (infective dose (ID)), or to calculate an LD_{20} or LD_{90}.

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3.2 Measuring Immune Responses

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CONTENTS

Introduction
Ex vivo reverse transcription of mRNA and PCR of cDNA using cytokine-specific primers
Immunohistology

INTRODUCTION

When analysing the immune response in experimentally infected animals, the goal is both to detect differences in the kinetics and magnitude of, for instance, cytokine expression in individual tissues, and to define the cellular localization of this response. More often than not, detection at the protein level is not feasible because the abundance of the target structure *in situ* is too small to be accessible to immunohistochemical or enzyme-linked immunosorbent assay (ELISA) methods. As an alternative strategy, procedures employing the polymerase chain reaction (PCR) to determine mRNA levels for cytokines and chemokines are outlined in this chapter. These methods are exquisitely sensitive, but have the distinct disadvantage that the cells expressing the mRNA cannot be identified directly. Immunohistology has long been the standard procedure for detecting molecules associated with individual cells within structurally intact tissue. This chapter therefore also describes immunohistological techniques that permit the localization of an induced immune response at the protein level *in situ*. A combination of both approaches will often lead to a complete picture of the nature of the infection-induced immune response.

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◆◆◆◆◆◆ *EX VIVO* REVERSE TRANSCRIPTION OF mRNA AND PCR OF cDNA USING CYTOKINE-SPECIFIC PRIMERS

General Precautions

When working with RNA, every attempt should be made to keep reagents and utensils free of RNase. This involves making buffers and stock reagents with diethylpyrocarbonate (DEPC) treated water, double-autoclaving all utensils and using disposable articles wherever possible. Gloves must be worn at all times and should be changed frequently. When working with RNA, all solutions, tubes, etc., should be kept on ice.

Cross-contamination is the biggest problem in all PCR procedures, and great care should be taken to avoid contamination of test samples with extraneous sources of DNA. Therefore, only use aerosol-resistent pipette tips, use separate pipettes for reagents and stock solutions, and use separate pipettes for PCR and RNA work. It helps to aliquot source reagents in a room away from where RNA extraction and PCR is performed, and RNA work-up and PCR pipetting should be done in separate rooms as well. Most importantly, pipetting amplicons (i.e. for electrophoresis) should be carried out with a set of entirely different pipettes (preferably old and used ones, so nobody else uses them) and in a completely different room. If contamination has occurred, it helps to clean surfaces and pipettes with a solution of 0.1 N HCl.

Harvesting Tissues

1. Remove tissues to be analysed under aseptic conditions. In kinetic studies, it is useful always to sample the same part of the organ (e.g. upper right liver lobe, lower left lung lobe). The size of the removed tissue should be roughly the same throughout the course of the experiment.
2. Place samples immediately into chilled lysis buffer (approximately 1 ml per 10–100 mg tissue):
   - 4 M guanidine–isothiocyanate (Merck)
   - 25 mM sodium citrate (pH 7)
   - 0.5% *N*-lauroylsarcosine (Sigma)
   - 100 mM 2-mercaptoethanol.

   Alternatively, commercially available lysis buffers may be used (e.g. TRIzol, Gibco BRL).
3. Samples can now be snap-frozen on liquid nitrogen and stored at –70°C until homogenization.
4. Alternatively, samples can now be homogenized using either an ultra-turrax at full speed or Teflon pestles and tight-fitting glass tubes. Use appropriate biosafety hoods when homogenizing infectious tissue. If you must reuse homogenizing tools, rinse at full speed (a) for 10 s in a beaker containing a large volume of dis-

tilled water, (b) for 10 s in a beaker containing a large volume of 95% ethanol, and (c) in lysis buffer, before additional tissue is homogenized.
5. After homogenization, store 500-µl aliquots in Eppendorf tubes for multiple work-ups. Mark tubes with indelible ink. Homogenized tissues in lysis buffer can be stored at –70°C indefinitely.

Preparation of RNA

1. To the tissue homogenates in lysis buffer (500 µl), add:
   - 50 µl 2 M sodium acetate (pH 4)
   - 500 µl acid phenol (water-saturated phenol)
   - 100 µl chloroform–isoamylalcohol mixture (49 : 1).
2. Vortex thoroughly. Let sit on ice for 15 min.
3. Centrifuge at 14 000*g* for 15 min at 4°C.
4. In steps of 100 µl, remove aequous supernatant (approximately 400 µl) to another labelled tube; take care not to disturb the interface.
5. Add 400 µl chilled isopropanol, vortex, and let precipitate for at least 2 h at –20°C. For some tissues, such as liver, it is advisable to perform two sequential phenol–chloroform extractions. Precipitates can be stored at –20°C indefinitely.

Some researchers prefer to resuspend and reprecipitate the RNA before performing reverse transcription. For this purpose, centrifuge samples for 15 min at 14 000*g* and remove the supernatant entirely. The RNA pellet should be white-translucent. Let the pellet dry (an evacuated desiccator works well) for 5–10 min, and resuspend in DEPC-treated water. Add approximately 100 µl water per 50 mg of tissue. Add 3 volumes of absolute ethanol and 1/10 volume of 3 M sodium acetate (pH 5.2). Reprecipitate at –20°C.

1. To determine the concentration of nucleic acid in the sample, centrifuge the precipitate at 14 000*g* for 15 min at 4°C.
2. Remove the supernatant, and add 500 µl chilled 70% ethanol (in DEPC-treated water).
3. Flick pellet briefly, and centrifuge at 14 000*g* for 15 min at 4°C.
4. Remove the supernatant and let the RNA pellet dry in an evacuated desiccator for 5–10 min.
5. Resuspend the RNA pellet in 100 µl DEPC-treated water and measure the optical density (OD) 260/280 of a 1 : 100 dilution (1 mg RNA has an OD of 1.0 at 260 nm):

Concentration of RNA (µg ml^{-1}) = OD reading × Dilution factor × 40

You may wish to check the integrity of the RNA on a formaldehyde gel at this point.

Reverse Transcription

1. Add 5 µg RNA to an Eppendorf tube, add 2 µg oligo-dT (12–18mer, Sigma), and make up the volume to 10 µl with DEPC-treated water. Be careful not to use too much RNA in the reverse transcription (RT) reaction.
2. Heat at 65°C for 10 min, then place on ice. While heating prepare the RT mixture (10 µl per sample):

   - 5 × first strand buffer 4 µl
   - 100 mM DTT 2 µl
   - 10 mM dNTPs 2 µl
   - RNAsin (40 000 U ml^{-1}; Promega) 1 µl
   - Superscript II RT (200 U ml^{-1}; Gibco BRL) 1 µl.

   Always add Superscript II RT last, and return the enzyme stock to the freezer immediately.
3. Add 10 µl RT mixture to each tube, after cooling. Vortex and quick-spin down.
4. Incubate at 37°C for 60–90 min.
5. Boil at 95°C for 2 min to terminate the reaction.
6. Dilute sample with distilled water (at this point, do *not* use DEPC-treated water), e.g. add 180 µl water. cDNA may be stored at –20°C indefinitely or for up to 2 weeks at 4°C.

Qualitative PCR

For primer selection, remember that primers should be complementary to sequences in separate exons, or should span exon–exon boundaries, in order to be mRNA/cDNA specific. 5'-Primers should contain sequences not more than 1000 bp removed from the poly-A-tail of the mRNA in order to avoid misrepresentation due to inefficient reverse transcription. Primers should be checked for unique complementarity using a computer-assisted search; for most purposes, annealing temperatures around or higher than 60°C will guarantee specificity.

- For each PCR, add to one tube:

10 mM dNTPS	0.5 µl
50 mM $MgCl_2$	1.25 µl
water	10.75 µl
10 × buffer	2.5 µl
TAQ polymerase (5 U μl^{-1}; Perkin-Elmer)	0.1 µl
primers (mix of sense and antisense), 1 µM	5 µl
cDNA	5 µl

- It is best first to put 5 µl of each primer mixture in the bottom of the tube, then make a mixture of the other PCR reagents for all reactions, and vortex thoroughly. Add 20 µl of this reaction mix to the side of each tube.

If you wish to perform a PCR for different cDNA samples at the same time, first put 5 µl of primer mixture to the PCR tubes, then make the PCR

mixture leaving out the cDNA, add 15 µl of the reaction mixture to one side of the tube and 5 µl of the cDNA to the other side. After closing and numbering the tubes, spin briefly to make sure all reagents mix at the bottom of the tube. If you use a thermal cycler without a heated lid, add one drop of mineral oil on top of the reagent mix to all tubes (you need not spin first; spinning will allow the aequous reagents to pass through the oil phase). Prepare a chart detailing the tube numbers with primer and cDNA contents.

The primers shown in Table 1 work with the following protocol:

- 95°C for 45 s
- 60°C for 45 s
- 72°C for 30 s
- 25–30 cycles for murine liver and spleen samples.

Electrophoresis and Detection of PCR Products

1. Prepare 2% agarose gel in 0.5 × tris-borate-EDTA (TBE) buffer. (You may add 0.5 µg ml^{-1} ethidium bromide at this point to visualize amplicons on an ultraviolet (UV) tray during electrophoresis, but a more even staining of the gel is obtained when soaking the gel *after* electrophoresis in 0.5 × TBE containing 0.5 µg ml^{-1} ethidium bromide.)
2. Add 5 µl of 5× loading buffer to each well of a microtitre plate.
3. Remove 15–20 µl PCR solution, mix with loading buffer in the wells, and add to individual slots in the gel. (You may use less PCR solution if you want to perform additional studies on the amplicon. A suitable control for the specificity of the amplicon involves a restriction enzyme digest of the PCR product resulting in the predicted pattern of bands after electrophoresis.)
4. Add molecular-weight markers to the first and last rows. If you have many samples, it is useful to double-comb the gels.
5. Run at 120 V for approximately 2–3 h, until the dye front reaches the bottom end of the gel.
6. Use any form of photodocumentation to visualize the bands on a UV transilluminator.

A typical example of results obtained with this type of protocol is shown in Fig. 1.

If no amplicon is obtained, repeat the PCR. If a given primer pair gives no product, try using a lower annealing temperature. Remember that some primers will not work however carefully they have been selected, and it may save time to construct new primer pairs. If no bands are visible even for the controls (β-actin, β_2-microglobulin, HPRT, etc.), the RT is usually at fault. Although only one reagent may have gone bad (i.e. has become contaminated with RNase), it is advisable to prepare new stocks of all reagents and to start again from the beginning.

Table 1. Primer sequences for qualitative RT-PCR*

Target mRNA	Sense primer	Amplicon size (bp)	Antisense primer
β_2-Microglobulin	ggCTCgCTCggTgACCCTAgTCTTT	300	TCTgCAggCgTATgTATCAgTCTCA
IL-1β	TCATgggATgATgATgATAACCTgCT	502	CCCATACTTTAggAAgACACggATT
IL-2	ATgTACAgCATgCAgCTCgCATCCTg	320	AgTCAAATCCAgAACATgCCgCAgAgg
IL-3	ATggTTCTTgCCAgCTCTACCACCA	592	gATAAgACATTTgATggCATAAAggA
IL-4	ACAAAAACACTTgAgAgAgATCAT	351	AgTAATCCATTTgCATgATgCTCTT
IL-6	CTggTgACAACCACggCCTTCCCTA	600	ATgCTTAggCATAACgCACTAggTT
IL-10	ACCTggTAgAAgTgATgCCCCAggCA	237	CTATgCAgTTgATgAAgATgTCAAA
M-CSF	AgTgAgggATTTTTgACCCAggAAgC	726	CTATACTggCAgTTCCACCTgTCTgT
TNFα	AgCCCACgTCgTAgCAAACCACCAA	446	ACACCCATTCCCTTCACAgAgCAAT
IFN-γ	gAAAgCCTAgAAAgTCTgAATAACT	388	ATCAgCAgCgACTCCTTTTCCgCTT

IFN, interferon; IL, interleukin; M-CSF, macrophage colony stimulating factor; TNF, tumour necrosis factor.

* From Ehlers *et al.* (1992).

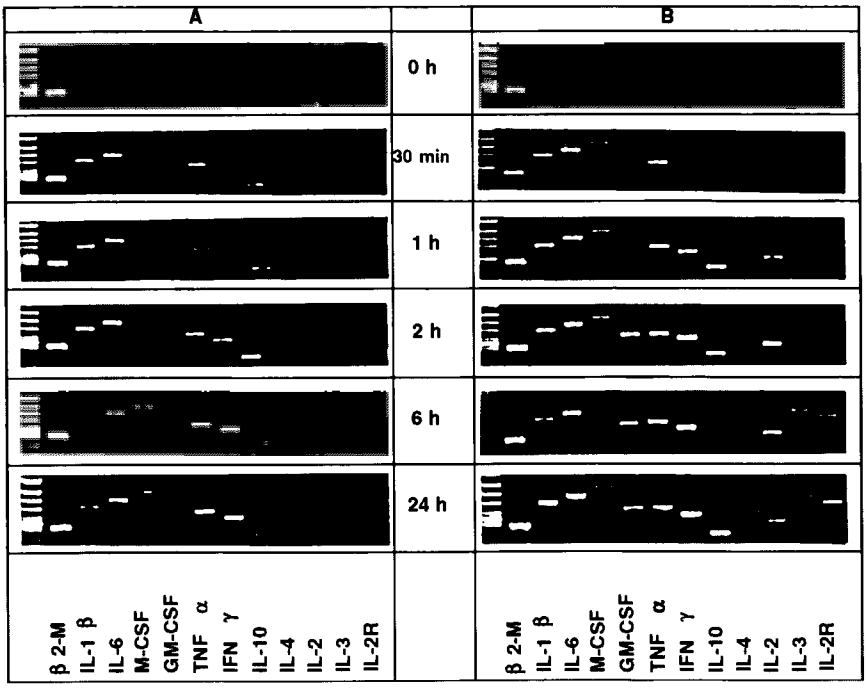

Figure 1. Qualitative RT-PCR of the livers of naive (A) and *Listeria*-immune (B) mice. C57BL/6 mice were challenged intravenously with 1×10^7 cfu *L. monocytogenes*, and liver samples were processed for RT-PCR at various time points after infection according to the protocol. (From Ehlers *et al.* (1992). Copyright © 1992 The American Association of Immunologists.)

Although all primers in Table 1 should work without problems under the conditions indicated, these conditions are not necessarily optimal. Small changes in $MgCl_2$, Taq-polymerase or dNTP concentrations and lowering of annealing temperatures may lead to extraneous bands. It sometimes helps to optimize conditions for each primer pair, and run PCR reactions for each primer pair individually, rather than for all primer pairs simultaneously.

Semiquantitative RT-PCR

The purpose of semiquantitative PCR is to compare RNA samples from different sources for their relative content of specific mRNAs. Since no absolute quantitation is involved, no exact calibration using *in vitro* synthesized RNA is necessary. However, only cDNAs normalized for their content of house-keeping genes (i.e. β_2-microglobulin or HPRT) may be compared directly to one another. Furthermore, comparison should always be done during the same PCR run, and the

types of semiquantitative PCR described will reproducibly discriminate between approximately three-fold differences only.

Using a competitor DNA control fragment

The advantage of this technique is that no additional equipment is necessary. You do need, however, a competitor DNA that will co-amplify in the PCR. Throughout the reaction, this competitor DNA fragment containing identical primer sequences competes with the cDNA for amplification, so that competitor/cDNA proportions are kept similar over a large number of cycles. However, in order to visualize amplicons by ethidium bromide, it is often necessary to use high cycle numbers so that even small amounts of contamination may become a problem. With the use of a control fragment containing all primer sequences in tandem array, contaminating the reagents with this fragment or an amplicon thereof results in additional bands for all primers used, even if you only use the fragment for quantitation of one cytokine. To limit contaminations, it helps to use different primer pairs for qualitative and semiquantitative PCR.

There are several ways to obtain a control DNA that will co-amplify in the PCR. One strategy involves running a PCR with extraneous DNA (e.g. chicken genomic DNA) in the presence of a selected primer pair at a low annealing temperature. This will give a number of amplicons, usually including one that is close in size to the amplicon of the cDNA. Excise the amplicon from the gel, purify it with any commercially available technique (e.g. Qiagen columns), and continue reamplifying and purifying until only one distinct amplicon is left. This control DNA may now be used to compete with the cDNA in the PCR.

It is, of course, more convenient to use only one single control DNA for all primer pairs. This involves cloning of an artificial DNA fragment that contains all the primer sequences in a tandem array. This is a lengthy procedure, but some researchers have agreed to make their constructs available to colleagues.

During the PCR, a constant amount of the control DNA is added to the PCR mixture. The correct amount will depend on the amount of amplifiable cDNA in the sample; if a lot of specific cDNA can be amplified, only very little competitor needs to be added; conversely, if a lot of specific cDNA can be amplified, a lot of competitor is needed to compete in the titration range. It is best first to determine the correct amount of competitor useful for your type of analysis.

1. Make step-wise dilutions of the cDNA to be analysed (do not make dilution steps smaller than three-fold). Preliminary experiments will determine the range in which the cDNA efficiently competes with the amount of control DNA chosen. Since the comparison is based on those titres that give equally dense bands of amplicons in the samples to be compared, you must first ensure that you are within the correct range.

2. Make the PCR mixture (μl per PCR reaction):

• 10 mM dNTPS	0.5 μl
• 50 mM $MgCl_2$	1.25 μl
• water	8.75 μl
• 10× buffer	2.5 μl
• TAQ polymerase (5 U μl^{-1})	0.1 μl
• 1 μM primer (mixture of sense and antisense)	5 μl
• competitor control DNA	2 μl.

(Table 2 gives a choice of primer pairs that work with the competitor DNA plasmid pMUS.)

3. Add 20 μl PCR mixture to each reaction tube.
4. Add 5 μl cDNA in step-wise dilutions.
5. Run the PCR and electrophoresis as above.

A typical result with this type of competitive PCR is shown in Fig. 2. Equal amounts of amplifiable cDNA were loaded as detected by a similar titre of β_2-microglobulin cDNA in all samples. However, since five-fold dilutions of cDNA are run in the presence of a constant amount of competitor DNA, a 25-fold difference in IFN-γ mRNA can be detected when samples A and B are compared.

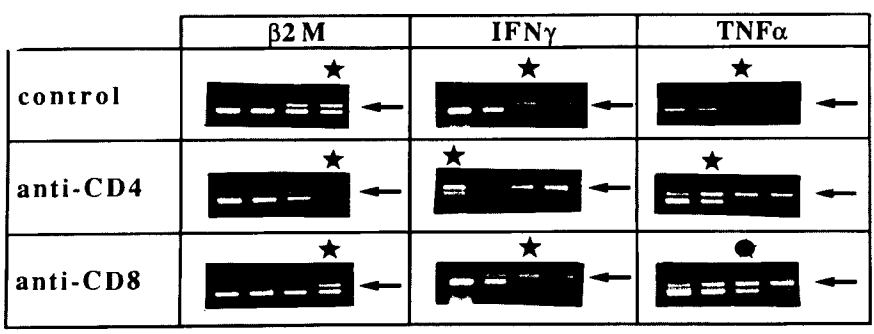

Figure 2. Semiquantitative RT-PCR for IFN-γ and TNFα in the livers of *Mycobacterium avium*-infected C57BL/6 mice depleted of T-cell subsets immediately after infection. C57BL/6 mice were intravenously infected with 2×10^5 *M. avium* SE 01 and treated intraperitoneally with saturating amounts of anti-CD4, anti-CD8 and control monoclonal antibody (mAb) immediately after infection and once weekly thereafter. Total RNA was prepared 3 weeks after infection from a caudal liver lobe and reverse transcribed. PCR was performed on 1 : 5 serially diluted cDNA in the presence of a constant amount of competitor control DNA fragment bearing the same primer sequences. The control amplicon is marked by an arrow. The dilutions at which equal amplification of control and cDNA were observed are marked with an asterisk. (From Hänsch *et al.* (1996). By permission of Oxford University Press.)

Table 2. Primer sequences for semiquantitative RT-PCR using a competitive DNA plasmid (pMUS)*

Target mRNA	Sense primer	Amplicon size (bp)	Antisense primer
IL-1α	CAgTTCTgCCATTgACCATC	218	TCTCACTgAAACTCAgCCgT
IL-1β	TTgACggACCCCAAAAgATg	204	AgAAggTgCTCATgTCCTCA
IL-2	gACACTTgTgCTCCTTgTCA	227	TCAATTCTgTggCCTgCTTg
IL-3	gACCCTCTCTgAggAATAAg	232	CTCCAgATCgTTAAggTggA
IL-4	TCggCATTTTgAACgAggTC	216	gAAAAgCCCgAAAgAgTCTC
IL-5	TCACCgAgCTCTgTTGgACAA	201	CCACACTTCTCTTTTTggCg
IL-6	gTTCTCTgggAAATCgTggA	208	TgTACTCCAggTAgCTATgg
TNFα	TCTCATCAgTTCTATggCCC	212	gggAgTAgACAAggTACAAC
IFN-γ	gCTCTgAgACAATgAACgCT	227	AAAgAgATAATCTggCTCTgC
GM-CSF	TgAACCTCCTggATgACATg	218	gTgTTTCACAgTCCgTTTCC
IL-2Rp55	gCAACTCCCATgACAAATCg	219	ATCCCggAATACACTCgTAg
β_2-Microglobulin	TgACCggCTTgTATgCTATC	222	CAgTgTgAgCCAggATATAg

IFN, interferon; IL, interleukin; GM-CSF, granulocyte–macrophage colony stimulating factor; TNF, tumour necrosis factor.

* From Shire, D. (1993).

Using oligonucleotide hybridization

When Southern blotting is used to detect the amplicons, it is possible to detect PCR products during the exponential phase of amplification, obviating the need for competitive PCR. In this case, direct comparison of the amount of product between samples is possible via comparison with a reference dilution standard. The latter is necessary to ensure that amplification is still proceeding in the exponential phase and has not yet reached a plateau. The optimum number of cycles will differ for each pair of specific primers used, and determination of the optimum number of cycles (i.e. the number that achieves a detectable amplicon concentration while still in the exponential phase of amplification) must be performed prior to semiquantitative cDNA analysis.

In principle, all primer pairs that work during qualitative PCR may be used for semiquantitative PCR. To reduce contamination with amplicons, however, it is suggested that you use different primer pairs for qualitative and semiquantitative purposes. The oligonucleotide probe used to detect the amplicon specifically should be complementary to approximately 20–30 bp of the amplified sequence. Table 3 gives a selection of primers and probes that work well together.

1. Perform the PCR exactly as described for qualitative PCR, but use cycle numbers within the exponential phase of amplification.
2. After the PCR is completed, load samples into a 2% agarose gel and run for 30 min at 150 V.
3. Denature gel in 500 ml buffer (1.5 M NaCl, 0.5 M NaOH) for 30 min with slight agitation in a plastic box with smooth surfaces.
4. Remove denaturing solution and rinse gel in deionized water.
5. Add 500 ml neutralizing solution (1.5 M NaCl, 1M Tris-HCl, pH 7.5) for 30–45 min at room temperature.
6. Use gloves for all subsequent steps. Cut nylon membrane (Hybond N+, Amersham) and wet by capillary action in 10 × SSC (0.15 M trisodium citrate, 1.5 M NaCl).
7. Trim gel to the size of membrane, and transfer DNA to the membrane by a standard blotting procedure (i.e. electroblotting or capillary blotting overnight). Take care to avoid bubbles.
8. Once transfer is complete, mark the nylon membrane and cross-link the membrane using a UV Stratalinker 1800 (Stratagene). Cross-linked blots can be kept sealed in a hybridization bag for up to 6 months.
9. For the detection of PCR products, a commercial oligolabelling and detection system (e.g. the Amersham ECL-kit) may be used. To prepare sufficient labelled probe for approximately 10 blots mix:
   - 100 pmol probe in water (for 20-mers, this corresponds to approximately 700 ng) 59 µl
   - fluorescein 5 µl

- cacodylate buffer 8 µl
- terminal transferase 8 µl.

It is helpful to have a master probe mix in aliquots of 100 pmol in 59 µl stored at –20°C ready for labelling.

10. Mix reagents and incubate at 37°C for 90 min. Store labelled probes at –20°C (for up to 6 months).
11. Prepare prehybridization solution:

    - 5 × SSC
    - 0.1% hybridization buffer component
    - 0.02% sodium dodecyl sulfate (SDS)
    - 20-fold dilution of liquid block solution.

    Combine all the components, make up to the required volume and mix well by stirring. The volume of buffer should be equivalent to 125 µl cm^{-2}. Prehybridization solution can be made ahead of time and stored in aliquots at –20°C for up to 3 months.
12. Place blot into the hybridization buffer and prehybridize for a minimum of 30 min at 42°C in a shaking water bath. Use a sealed hybridization bag for hybridization; avoid bubbles.
13. After prehybridizing the blot, add 7 ng labelled probe per millilitre of prehybridizing solution. It is best to add the required amount of labelled probe to 0.5 ml of prehybridizing solution and add the entire volume to the prehybridizing bag. Reseal the bag, avoiding bubbles. Incubate at 42°C for 2 h in a shaking water bath. Make up the blocking buffer (buffer 1):

    - 0.15 M NaCl
    - 0.1 M Tris base (adjusted to pH 7.5 with concentrated HCl and made up to 1 l with sterile water)
    - plus 1/20 of liquid block reagent added and stirred for 30 min.

14. Remove blots from the bags and wash twice with an excess volume of 5 × SSC, 0.1% SDS at room temperature for 5 min, followed by two additional washes with 1 × SSC, 0.1% SDS at 45°C for 15 min while agitating the plastic container.
15. Wash blots in buffer 1 for 1 min.
16. Discard buffer 1 and replace with blocking buffer (i.e. buffer 1 plus 1/20 of liquid block reagent added and stirred for 30 min prior to use).
17. Incubate for at least 30 min.
18. Rinse blot briefly in buffer 1.
19. Dilute the antifluorescein HRP conjugate 1000-fold in buffer 2:

    - 0.4 M NaCl
    - 0.1 M Tris base
    - 0.5% (w/v) bovine serum albumin (BSA) (fraction V) (adjusted to pH 7.5 in a final volume of 1 l).

20. Incubate the blot in the diluted antibody conjugate solution for 30 min.
21. Place filters in a clean container and rinse with an excess of buffer 2 (without BSA) for 5 min; repeat this wash another three times. From now on work with reasonable speed, as the ECL™ reaction proceeds without a lag phase. Wear powder-free gloves to avoid blank patches on autoradiograms.
22. Mix equal volumes of detection solution 1 and detection solution 2 to give sufficient reagent to cover the blot (125 µl cm^{-2} is sufficient).
23. Drain the excess buffer from washed blots and place them on a sheet of SaranWrap, DNA side up.
24. Add the detection buffer directly to the blot on the side carrying the DNA. The membrane must be moist at all times.
25. Incubate for exactly 1 min at room temperature.
26. Drain off excess detection buffer and wrap blots in SaranWrap. Remove all air pockets.
27. Place the blot DNA side up in the film cassette.
28. In a dark room, place a sheet of autoradiography film (Hyperfilm-ECL) on top of the blots. Close the cassette and expose the film for 1–4 h.
29. Obtain several exposures to ensure you are within the linear range of the film.

It is now possible to compare signals generated from the amplified cDNA with a standard curve generated from simultaneously amplified step-wise dilutions of cDNA obtained from samples that have a high amount of specific cytokine mRNA (e.g. from *in vitro* stimulated cell lines or clones).

For semiquantitative comparisons to be meaningful, hypoxanthine-guanine phosphoribosyl transferase (HPRT) signals should not vary more than three-fold between groups in any given experiment.

At this point, you may wish to quantify the chemiluminescent signals by using a scanning device to define more accurately any differences in mRNA levels. In this way, it is possible to plot the fold increase induced during infection by assigning to the background level in uninfected controls an arbitrary value of 1 (Fig. 3).

Limitations

Any RT-PCR measures steady state mRNA levels, i.e. quantitative differences may only be found when there is induced or repressed transcription of mRNA or increased or repressed turnover of mRNA. Whenever the amount of cytokine secreted is highly regulated by translational modifications (as is the case with, for example, tumor necrosis factor α and transforming growth factor β (TNFα and TGFβ)) it may be difficult to measure mRNA differences, although at the protein level major differences are observed. However, it is true that, where there is no mRNA, there usually

Table 3. Primer and probe sequences for semiquantitative RT-PCR using oligonucleotide probes*

Target mRNA	Sense primer Antisense primer	Amplicon size (bp)	Probe
HPRT	gTTggATACAggCCAgACTTTgTTg gATTCAACTTgCgCTCATCTTAggC	165	gTTgTTggATATgCCCTTGAC
IL-1β	gggATgATgATgATAACCTg TTgTCgTTgCTTggTTCTCCT	196	CAgCTgCACTACAggCTCCg
IL-2	gAgTCAAATCCAgAACATgCC TCCACTTCAAgCTCTACAg	248	CTCCCCAggATgCTCACCTTC
IL-4	gAATgTACCAggAgCCATATC CTCAgTACTACgAgAATCCA	384	AgggCTTCCAAggTgCTTCgCA
IL-5	gACAAgCAATgAgACACgATgAgg gAACTCTTgCAggTAATCCAgg	235	gggggTACTgTggAAATgCTAT
IL-6	TTCCATCCAgTTgCCTTCTTgg CTTCATgTACTCCAggTAg	360	ACTTCACAAgTCCggAgA
IL-10	CgggAAgACAATAACTg CATTTCCgATAAggCTTgg	187	ggACTgCCTTCAgCCAggTgAAgA
IL-12 p40	CgTgCTCATggCTggTgCAAAg CTTCATCTgCAAgTTCTTgggC	314	TCTgTCTgCAgAgAAggTCACA

Table 3. (*contd*)

Target mRNA	Sense primer Antisense primer	Amplicon size (bp)	Probe
TNFα	gATCTCAAAgACAACCAACTAgTg CTCCAgCTggAAgACTCCTCCCAg	255	CCCgACTACgTgCTCCTCACC
IFN-γ	AACgCTACACACTgCATCTTgg gACTTCAAAgAgTCTgAgg	238	ggAggAACTggCAAAAggA
iNOS	CTggAggAgCTCCTgCCTCATg gCAgCATCCCCTCTgATggTg	440	CTggATgAgCTCATCTTTgCC
GM-CSF	TgTggTCTACAgCCTCTCAgCAC CAAAggggATATCAgTCAgAAAgT	368	CgAgTTCTCCTTCAAgAAgC
MIP-1α	gCAgCAgCgAgTACCAgTCCC ggCATTCAgTTCCAggTCAg	343	gCCTgCTgCTTCTCCTACAg
MIP-2	gCCCCTCCCACCTgCCggCTCC CTgAACCAgggggggTTCAggg	258	gTgTgACgCCCCCAggACCCC
MCP-1	AgAgAgCCAgACggAggAAg gTCACACTggTCACTCCTAC	519	CCAgATgCAgTTAACgCCCC
IP-10	TgAACCCAAgTgCTgCCgTC CTCAggACCATggCTTgACCATC	483	CCCTgCgAgCCTATCCTgCCC

GM-CSF, granulocyte–macrophage colony stimulating factor; HPRT, hypoxanthine-guanine phosphoribosyl transferase; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; IP, interferon-γ inducible protein; MIP, macrophage inflammatory protein; MCP, macrophage chemotactic protein; TNF, tumour necrosis factor.
* Data from Svetic *et al.* (1991), Wynn *et al.* (1993), Sonouche *et al.* (1994) and Rhoades *et al.* (1995).

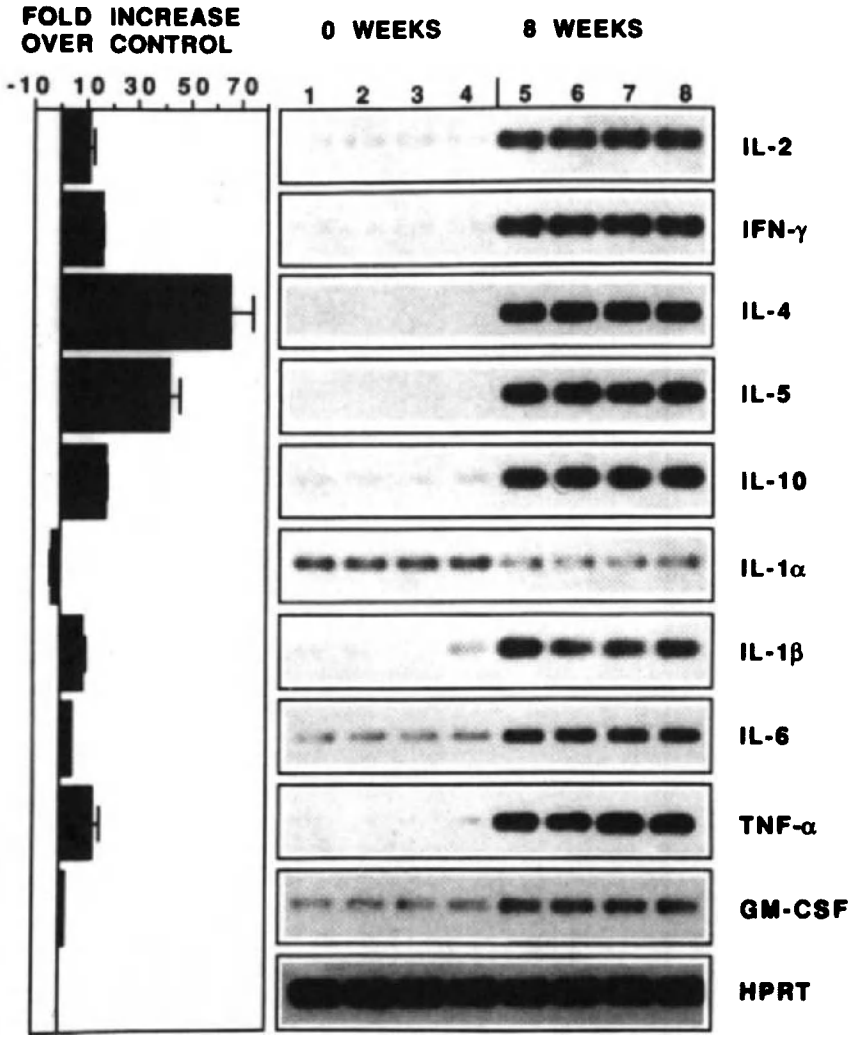

Figure 3. Pattern of cytokine mRNA expression in the livers of mice infected with *Schistosoma mansoni*. C3H/HeN mice were infected with 25±3 cercariae of *S. mansoni* by percutaneous exposure of the tail. Animals were sacrificed 8 weeks later. Total RNA was isolated from livers and assayed for expression of cytokine mRNA by RT-PCR. The Southern blots of amplified PCR products are shown on the right-hand side. Four animals per group were analysed individually: lanes 1–4, uninfected mice; lanes 5–8, infected mice. The panel on the left-hand side represents the fold increases in cytokine gene expression. All values are expressed relative to uninfected controls, which are given an arbitrary value of 1 and represent the mean and standard deviation of cytokine mRNA levels from the livers of the four individual mice. (From Wynn et al. (1993). Copyright © 1993 The American Association of Immunologists.)

is no protein; thus RT-PCR is always a good indicator of what cytokine *not* to look for by ELISA in tissue homogenates or by immunohistochemical analyses.

Remember that the kinetics of induction for mRNA species vary tremendously, and always precede peaks of protein production.

Therefore, a detailed kinetic study is often warranted to define where best to look for differences when comparing experimental settings.

Always be aware that these procedures allow for the relative quantitation of mRNA for any given cytokine, but do not allow for comparison of mRNA levels between different cytokines. Also, when using competitor DNA, titration end-points obtained in one experiment need not be the same as in another experiment, due to the variablility in the concentration of the control plasmid DNA employed.

Some good examples of *ex vivo* qualitative and semiquantitative RT-PCR results in different experimental settings are given in the Further Reading section at the end of this chapter.

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Tissue Preparation

Frozen tissues

1. Remove tissues and place into a flat-bottomed cylindrical sealable plastic tube filled with sterile phosphate buffered saline (PBS) or normal saline in order to cover fully the tissue sample. Antigenicity is best preserved under these conditions, although some researchers prefer commercial embedding media (e.g. Cry-M-bed, Bright Instruments).
2. With long forceps, immediately place the sealed container in an upright position into liquid nitrogen until frozen. It is important to snap-freeze fresh specimens to prevent the formation of ice crystals in the tissue. These will appear as slits or fracture lines and give rise to artefactual staining. Containers may be stored at –70°C indefinitely. Tissues should always be maintained in the frozen state. If they are allowed to thaw, refreezing will result in extensive damage to the tissue, as well as loss of antigenicity.
3. Prepare cryostat sections of 4–5 µm thickness.
4. Air dry sections for 4–24 h. Fix for 15–30 min in acetone followed by 15–30 min in chloroform. (If the cryostat sections are not meant to be stained the following day, it is advised to also air dry them for 4–24 h, fix them for 10 min in acetone and store them at –70°C. When needed, thaw the sections covered with a paper towel in order to prevent water condensation on the frozen slides, and fix in acetone and chloroform, as detailed above.)
5. For some staining procedures (particularly to detect cytokines), fixing of specimens with paraformaldehyde/saponin is preferred. For this purpose, fix the air-dried slides for 15 min in 4% paraformaldehyde in PBS without NaCl (pH 7.4–7.6), followed by a 15-min treatment in 0.1% saponin/PBS to remove cholesterol from the membranes. Wash thoroughly with PBS before immunostaining.

1. Prepare sections from paraffin blocks onto precleaned, grease-free slides.
2. Dewax routinely processed paraffin sections by submerging them for 10 min in xylene followed by 10 min in acetone and 10 min in a 1 : 1 mixture of acetone and Tris-buffered saline (TBS: 50 mM Tris, 150 mM NaCl, pH 7.5). Keep the sections in TBS until staining. In most cases, it will be necessary to try several approaches for antigen retrieval.
3. Deparaffinate the sections for 5 min in xylene and rehydrate them in a series of acetones at 5-min intervals (100%, 70%, 40%). Rinse under running tap water.

Microwave pretreatment

1. Transfer the slides to a plastic staining jar filled with 10 mM sodium citrate buffer (pH 6).
2. Place the plastic staining jars into a microwave oven and heat for 5 min at 720 W. After 5 min it is essential that the staining jar be refilled with buffer to prevent the specimens from drying out. The frequency of the heating steps depends on the embedding procedure performed previously and must be optimized for each laboratory individually. Thus, the time of formalin fixation and the quality of the paraffin may influence antigen retrieval. In most instances, heating the sections 3–6 times for 5 min will be sufficient to give good results.
3. After microwaving, let the slides cool down in the staining jar for approximately 20 min at room temperature, and rinse briefly in TBS before proceeding with immunostaining.

Pressure cooker pretreatment

1. Fill a normal household pressure cooker with enough 10 mM sodium citrate buffer to cover the slides (approximately 2 l). Bring the buffer to a boil before submerging the slides, close the lid and heat until the top pressure is reached. Boil the sections at this pressure for 1–10 min. As with microwaving, the boiling time must be optimized depending on the embedding procedure used, because variables such as time of formalin fixation, quality of paraffin and section thickness may influence antigen retrieval.
2. Cool the pressure cooker under running cold water. (Take extreme care when performing this step and follow exactly the manufacturer's instructions for opening the cooker.)
3. Transfer the sections to TBS, and wash in TBS three times before proceeding with immunostaining.

Staining Procedure: Peroxidase Method

For a more detailed outline of various immunoenzymatic methods see the chapter by Seitzer and Gerdes in Section III. When working with mouse tissue, the primary antibody is most likely to be a rat monoclonal antibody or a rabbit polyclonal antiserum. Below, we give a description of primary and secondary reagents necessary for the peroxidase method, which should be a useful guide when adapting other protocols for use in animal tissues.

All incubations with antibodies are performed in a level humid chamber at room temperature, using approximately 100 µl of antibody solution. Excess humidity should be avoided, since condensation of water onto the slides will interfere with the staining reaction. On the other hand, insufficient humidity will dry out the antibody on the sections, resulting in false-positive staining. Drying is usually most apparent at the edges of the sections (rim effect). Between incubations, wash the slides twice in TBS in staining jars and drain off excess fluid by capillary action using a paper towel.

1. Always include negative controls without primary antibody and with an irrelevant primary antibody of the same immunoglobulin class (or with preimmune serum). The specificity of the primary antibody can be analysed by neutralization experiments in which blocking of antibody binding results in negative staining. For this purpose, preincubate the antibody with recombinant or purified antigen for 30 min at 37°C before applying it to the slides.
2. Before incubating the slides with primary antibody, block endogenous peroxidase activity in the tissue by preincubating the slides for 20 min in a light-protected staining jar with 1% H_2O_2 in TBS.
3. Add the rabbit primary antiserum or rat monoclonal antibody in the appropriate dilution in TBS containing 10% fetal calf serum (FCS). (To find the appropriate concentration of primary antibody, perform three-fold serial dilutions to optimize the ratio of background and specific staining.) Incubate for 30 min.
4. Add the first secondary peroxidase conjugated goat anti-rabbit immunoglobulin G (IgG) antibody (Dianova; diluted 1 : 30 in TBS/10% FCS and 20% mouse serum). When a rat monoclonal antibody is used as the primary-step reagent, add a peroxidase conjugated rabbit–anti-rat-IgG antibody (Dianova, diluted 1 : 30 in TBS/10% FCS and 20% mouse serum). The optimal concentration of secondary reagents is usually indicated by the manufacturer.
5. Incubate for 30 min.
6. Add the second secondary antibody: peroxidase conjugated rabbit–anti-goat-IgG, or peroxidase conjugated goat–anti-rabbit-IgG (Dianova; diluted 1 : 50 in TBS/10% FCS) depending on the primary and first secondary reagents used. Incubate for another 30 min.

7. Prepare the developing buffer by dissolving 6 mg 3,3′-diaminobenzidine tetrahydrochloride (Sigma) in 10 ml TBS. Add 100 µl H_2O_2 directly before use. Mix well.
8. Incubate the slides with developing buffer in the dark in the humid chamber for 3–15 min. The degree of development can be checked microscopically and is terminated at the desired point.
9. Counterstain with haematoxylin for 90–105 s. Haematoxylin stock solution is prepared from 1 g haematoxylin (Merck 4305), 0.2 g $NaJO_3$ (Merck 6525), 50 g aluminium potassium sulfate dodecahydrate ($KaI(SO_4)_2 \cdot 12\,H_2O$; (Merck 507 A 95744) in 1 l distilled water. After these reagents are dissolved, add 50 g chloral hydrate (Merck 2425) and 1 g citric acid (Merck 244). Before staining, pass the solution through a sterile filter. Haematoxylin solution may be reused a few times.
10. Rinse the slides in running tap water and leave in tap water for 5 min before mounting with prewarmed (56°C) Kaiser's glycerol–gelatine.

Typical results obtained using this method are shown in Plate 6 and described in the references given in the Further Reading section at the end of this chapter.

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Further Reading

Qualitative and semiquantitative RT-PCR results

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For a more extensive description of standard molecular biology techniques such as electrophoresis and Southern blotting, see the following manuals:

Perbal, B. (1988). *A Practical Guide to Molecular Cloning*. Wiley, New York.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

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List of Suppliers

Amersham
2636 South Clearbrook Drive
Arlington Heights
IL 60005, USA

Tel.: +1 800 323 9750
Fax: +1 800 228 8735

Hybridization equipment, chemiluminescent detection.

Bright Instruments Comp.
St. Margaret's Way
Stokeley Meadows
Huntington
Cambridgeshire PE186EB, UK

Tel.: +44 (0)1480 454528
Fax: +44 (0)1480 456031

Embedding solution for frozen tissue.

Dianova
P.O. Box 301250
20305 Hamburg
Germany

Tel.: +49 40 450670
Fax: +49 40 4506 7490

Monoclonal antibodies against murine/human tissue antigens.

Genzyme
1 Kendall Square
Cambridge
MA 02139-1562, USA

Tel.: +1 617 252 7500
Fax: +1 617 374 7300

Monoclonal antibodies against murine/human tissue antigens and cytokines.

Gibco BRL Life Technologies
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF, UK

Tel.: +44 (0)141 814 6100
Fax: +44 (0)141 814 6317

Molecular biology reagents (enzymes, molecular-weight markers, nucleotides).

Merck Co.
EM Inc.
5 Skyline Drive
Hawthorne,
NY 10532, USA

Tel.: +1 914 592 4660
Fax: +1 914 592 9469

Industrial chemicals.

Merck Co.
EM Science
480 Democrat Road
Gibbstown
NJ 08027, USA

Tel.: +1 609 423 6300
Fax: +1 609 423 4891

Chemical reagents.

Perkin-Elmer
850 Lincoln Center Drive
Foster City
CA 94404, USA

Tel.: +1 800 345 5224
Fax: +1 415 572 2743

PCR technology and PCR reagents.

Pharmingen
10975 Torreyana Road
San Diego
CA 92121, USA

Tel.: +1 619 677 7737
Fax: +1 619 677 7749

Monoclonal antibodies (against cytokines and murine/human tissue antigens).

Promega
2800 Woods Hollow Road
Madison
WI 53711-5399, USA

Tel.: +1 608 274 4330
Fax: +1 608 277 2516

Molecular biology reagents (restriction enzymes, RNase inhibitors).

Sigma Chemical Co.
P.O. Box 14508
St Louis
MI 63178-9916, USA

Tel.: +1 314 771 5750
Fax: +1 314 771 5757

Chemicals and biochemicals.

Stratagene
11011 North Torrey
Pines Road
La Jolla
CA 92037, USA

Tel.: +1 619 535 5400
Fax: +1 619 535 0034

Molecular biology, nucleic acid transfer and hybridization.

4 Specific Models

4.1 Murine Model of Tuberculosis

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Infection protocols
Culturing and infecting macrophages
Identifying key proteins recognized by immune T cells
Following the course of infection
Delayed-type hypersensitivity and non-specific resistance
Measuring the chemokine/cytokine response *in vivo*

◆◆◆◆◆◆ HANDLING AND CULTIVATION OF MYCOBACTERIA

The isolation and cultivation of *Mycobacterium tuberculosis* in both the clinical and research laboratory setting has been well described in numerous publications and books (Vestal, 1975; Youmans, 1979; Kubica and Wayne, 1984; Bloom, 1994; Rom and Garay, 1995). The methods described in many of these references have been in use for many years and have changed very little. For example, many laboratories routinely cultivate *M. tuberculosis* cultures in Proskauer and Beck liquid medium, which is a modified medium first developed by Youmans and Karlson in 1947. However, in this chapter we do not attempt to outline all the well-established methodologies that have been used by various investigators, but instead we concentrate on those utilized in our own laboratory for the cultivation of *M. tuberculosis* for research purposes.

In our laboratory we routinely cultivate strains of *M. tuberculosis* for use in the following activities: (a) the *in vivo* determination of disease host response and pathogenesis, primarily using a murine airborne infection

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model; (b) the *in vivo* determination of bactericidal effectiveness of novel immunotherapy, vaccination or chemotherapeutic agents using both the murine and guinea-pig airborne infection models; and (c) the *in vitro* testing of potential chemotherapeutic agents using a murine bone marrow derived macrophage infection model. Each of these procedures requires the cultivation of both 'laboratory strains' (such as Erdman, H37Rv) and newly acquired clinical isolates of *M. tuberculosis*.

While small inocula of *M. tuberculosis* can be handled safely in appropriate 'class II' biosafety cabinets, any larger operations require stringent BL-3 biosafety conditions. Although everyone seems to have their own definitions of this level, our recommended 'minimum requirements' are a facility with shower in/shower out (which also avoids transmission of mouse viruses), a safety cabinet, a glove box, air filtered both in and out through HEPA filters, and a graduated air flow handling system (a second, back-up system is good, but not essential). Animals should be kept in separate rooms away from equipment, because of animal bedding dust. Personnel should wear protective 'overclothing' or surgical scrubs (we prefer the latter; overclothes heat you up quickly and can also contribute to a feeling of claustrophobia), and should be skin tested about every 6 months. BCG (Bacille Calmette-Guérin) vaccinated personnel should consider having a chest X-ray every 1–2 years.

Industrial rather than surgical masks should be worn at all times. Infection procedures, tissue homogenization and plating can all potentially create significant aerosols, which, other than needle stick injuries, are the primary source of danger.

Each BL-3 should have an exposure control plan in place before starting operation, and all personnel should be familiar with this. In our experience accidents are very rare, but when they do occur often as not it is due to some mental preoccupation rather than a technical lapse. In other words, only work under BL-3 conditions when your mind can be kept completely on the task at hand.

Wherever possible, BL-3 laboratories should be stand-alone or part of a larger animal facility. If there are regular open laboratories on floors above the BL-3, then the air-handling systems should be very carefully checked on regular occasions (however, we do not recommend this type of architectural arrangement). If the facility includes any form of aerosol-generating device, this should be kept separately in a room under the lowest air pressure relative to the rest of the BL-3 facility.

Receiving New Cultures

The cultivation of *M. tuberculosis* in our laboratory begins with the receipt of clinical isolates from other research institutions or hospitals. When shipments arrive, the shipping container should be examined to make sure that it has not been damaged while in transit. All aetiological agents such as tuberculosis need to be shipped (within the USA) in accordance with the interstate quarantine regulations (Federal Register, Title 42, Chapter 1, part 72, revised 30 July 1972). The shipping container should

consist of an outer heavy cardboard shipping box, with adequate packing to absorb any crushing. Inside this box should be a leak-proof canister (such as heavy polypropylene biocontainment canisters with rubber seals) with absorbent packing material inside. Within this canister there should reside an additional canister with more absorbent material and the tubes containing the tuberculosis isolates on Lowenstein–Jensen slants. The outside of the shipping container should have all the appropriate biohazardous materials markings, as well as addresses and phone numbers of the shipper and recipient. The shipping box should also contain appropriate documentation describing the contents as per strains included and possible drug resistance data.

Upon receipt and initial inspection of the package it should be taken to an appropriate biosafety level 3 (BL-3) containment room or facility. The technician wearing appropriate facility-specific clothing and protective respirators should open the package within a biological safety cabinet. The package is carefully opened and examined for any damage or spillage onto the absorbent materials. If leakage has occurred the materials are thoroughly soaked with disinfectant (5% Lysol or Amphyl) and allowed to stand for 30 min before proceeding. If the interior tubes are damaged it is best carefully to place all materials in autoclave bags and autoclave immediately. Wipe down all work surfaces with disinfectant, and notify the sender of the condition of the shipment. If the contents of the package are intact, check what has been received versus the documentation which came with the package.

Cultivation

It is essential that stock cultures of *M. tuberculosis* used in research experiments should be standardized in terms of the culture medium as well as the culture conditions (initial inocula, temperature, aeration, agitation, subculture times, and so on). This is important because it ensures that each individual isolate has been cultivated under similar physiological conditions during growth, which is critical if you plan to compare data between strains and experiments.

Each laboratory has its own favourite broth mediums; we prefer Proskauer and Beck (Youmans and Karlson, 1947), or glycine alanine salts (GAS) broth. Both these liquid media are simple minimal salt solutions with glycerol as a carbon source. Our approach limits the amount of extraneous additives in our cultures such as those found in other broth media such as 7H9 broth, which requires enrichment with oleic acid–albumin dextrose complex (OADC). This is particularly important when trying to isolate secreted proteins or metabolites from a myocobacterial culture so as to avoid contaminating proteins from the culture medium.

Mycobacteria species in general, and *M. tuberculosis* in particular, have a tendency to clump or raft when cultivated in liquid broth media. This is a problem which is overcome by the addition of the detergent Tween 80 to the culture, which disperses the bacilli and provides a smooth, even suspension. When cultures are grown in Tween-containing media there can

be a more accurate determination of the true number of viable bacilli by optical absorbance or serial dilution plating on solid media such as 7H11 agar.

There has been some discussion as to the merits of adding detergents to the culture medium, as this may affect the pathogenicity or viability of the bacilli (Davis and Dubos, 1948; Dubos and Middlebrook, 1948; Dubos, 1950; Collins *et al.*, 1974). We recognize this concern, but feel that in order to achieve dispersed cultures free from large clumps, the addition of Tween is a necessary evil. For example, the necessity for smooth and evenly dispersed cultures is critical for the accurate delivery of bacilli for *in vivo* studies when the infection is given by aerosol. The aerosol device utilizes a glass venturi system to form tiny droplets containing the bacilli, which are then delivered to a chamber containing the test animals over a specified period of time. If inocula from different isolates with different degrees of clumping were to be used, the data generated would be meaningless because of the wide variation in uptake, as well as the variation in particle size (i.e. size of rafts). On the other hand, with smooth, evenly dispersed cultures the uptake parameters are highly reproducible.

Another critical parameter is that all cultures used in biological experiments should be harvested at the same phase of growth. We like to harvest broth cultures when they are still in the log growth phase, but before the culture enters into the late log growth/stationary phase. At this point the culture is at its optimum concentration and viability. Cultures harvested at this time routinely have colony forming unit (cfu) counts of 5×10^7 up to a maximum of 10^9 per millilitre of broth. Higher concentrations are not recommended; when the culture contains in the region of 10^{10} cfu ml^{-1} there is more clumping of the bacilli and many bacilli are probably dead. If required, the presence of non-viable bacilli in cultures can be detected by the presence of autolytic enzymes such as isocitrate dehydrogenase.

As described above, all manipulations with *M. tuberculosis* should be done in a biological safety cabinet (class II) within a BL-3 facility. The establishment of a primary seed culture collection or repository is essential in maintaining the integrity of the individual clinical isolates as they are received. Proper documentation as to the source, date received, accession number, strain name, history of isolate, drug susceptibility/resistance and virulence data should be maintained in some form of database. When a clinical isolate is received and unpacked, it first needs to be evaluated to see whether there is sufficient growth for immediate subculture. In most instances clinical isolates of *M. tuberculosis* arrive in the research laboratory on Lowenstein–Jensen (LJ) slants (Jensen 1932), but there are occasions when samples arrive in sealed serum vials containing broth cultures (1.0–2.0 ml).

If cultures arrive on LJ slants they usually have been incubated prior to shipment and have sufficient colony growth to allow immediate subculture into broth. Occasionally there is very little visible growth and these slants should be further incubated at 37°C for 1–2 weeks to establish visible colonies. Samples that have been shipped as broth cultures are usually at a high cfu density and can be subcultured directly into broth media. It

is generally more desirable to ship LJ slants because of the reduced risk of leakage while the package is in transit.

The first step in the subculture of clinical isolates is the preparation of the work area in the biological safety cabinet. The cabinet is turned on and allowed to run for at least 20 min to allow the blower motors to reach optimum operating speed and allow the air balance to be achieved. The interior work surfaces are then disinfected prior to use with 5% Lysol or Amphyl solution followed by 70% ethanol. All cleaning materials are deposited into a small autoclave bag, which is then taped to the inside wall of the cabinet. This bag is used as a waste bag for soiled gloves, Kimwipes, etc., while working in the cabinet, and is closed and removed when work is complete. The work surface within the cabinet is covered with bench blotters (i.e. plastic-backed paper towelling) which have been sprayed with disinfectant solution (Lysol or Amphyl). This is done to reduce the potential hazard resulting from unnoticed spatters that may occur during manipulations. A metal pipette boat with 2–3 cm of 5% Lysol is placed in a convenient working location for the technician (usually in the centre toward the back, but not too close or over the air returns of the cabinet). All materials that will be needed for manipulations, such as sterile pipettes, inoculation loops, sterile tubes, flasks and extra gloves, should be located close to the cabinet on a bench or laboratory cart. When all materials are assembled, work can proceed.

To establish broth seed cultures we use sterile 150×25 mm screw-capped culture tubes that have been prefilled with 20 ml of either Proskauer and Beck or GAS liquid medium supplemented with 0.05% Tween 80. Each tube contains a very small plastic-coated magnetic stir bar for culture aeration and agitation. Using a sterile disposable inoculating loop, a sample from the LJ slant is inoculated into the broth within the tube. It is important only to inoculate one loop; if you seed the culture with an overly heavy amount of bacilli the clumps will not dissipate and there is the potential of having a larger amount of dead bacilli in the seed stock upon harvest. The inoculation loop is then discarded carefully into the pipette boat containing the Lysol. The reason for using plastic disposable loops is that this minimizes the potential aerosol hazard that may occur with flaming a metal inoculation loop. Metal loops can be used, but should first be speared into a 70% ethanol sand bath to remove any remaining material before being flamed in an electric loop incinerator.

With broth samples, the top of the vial is first swabbed with 70% ethanol and then flamed with a Bunsen burner to sterilize. A 1-cm^3 tuberculin syringe fitted with a 26-gauge needle is then used to carefully remove 1.0–2.0 ml of the inoculum from the vial. The inoculum is then carefully injected down the side of the culture tube, taking care not to create an aerosol. Each inoculated tube is labelled with the following information: species (*M. tuberculosis*), strain, medium, date, technician and drug resistance (yes/no).

The seeded culture tubes are placed carefully into a plastic test-tube rack which has been secured on top of a magnetic stir plate within a 37°C incubator. The original LJ slants are deposited in a 4°C refrigerator within the biosafety laboratory and retained. All instruments used during

subculture are wiped down with disinfectant prior to removal from the cabinet. All waste materials are bagged in autoclave bags and removed immediately for sterilization in a steam autoclave (121°C at 15 lb inch^2 for 40 min). The interior surfaces of the biological safety cabinet are disinfected thoroughly with 5% Lysol followed by 70% ethanol.

The seeded cultures are checked twice a week for growth and possible contamination. Usually the cultures reach a density of 5×10^7 to 5×10^8 within 1–2 weeks after initial seeding. With experience, a trained technician can visually estimate the approximate cfu growth within a tube of broth. In general, a tube with less than 10^6 cfu ml^{-1} is clear and growth cannot be detected visually. At 10^7 cfu ml^{-1} there is a discernible cloudiness that can be seen. When the culture reaches 10^8 cfu ml^{-1} there is a very hazy growth, and printed text can be detected when placed behind the tube but individual letters cannot be visualized. A culture that has reached 10^9 cfu ml^{-1} or greater is very turbid/milky and some clumping or 'stringy' growth may be detected (resulting from the Tween being metabolized by the bacilli).

An optical density measurement at A580 can also be made to determine the relative concentration. When the cultures reach mid–late log growth (approximately 10^8 cfu ml^{-1}) they are subcultured into 150 ml of fresh broth in disposable 250-ml polycarbonate Erlenmeyer flasks with screw-type closures (Corning 25600). The flasks are labelled as above and incubated at 37°C on orbital shakers (slow speed with gentle agitation), or stationary with gentle swirling twice weekly for an additional 1–2 weeks. When the density has once again reached approximately 1×10^8 to 5×10^8 cfu ml^{-1} the culture is removed from the incubator to be aliquoted into serum vials for laboratory seed cultures. The mycobacterial culture is aliquoted in volumes of 1.5 ml into 2 ml sterile serum vials fitted with sterile butyl rubber stoppers; the stoppers are then crimped into place with sterile aluminium seals (Wheaton Glass). The vials are disinfected by immersion in 70% ethanol and then allowed to air dry. Each vial is then labelled with small Avery labels printed with information about the species, strain, date (lot), medium and technician. The vials are then placed in labelled storage boxes, which are then placed in a –70°C freezer for long-term storage.

After the vials have been frozen, a random sampling of vials from the storage box is taken out the following day for the determination of the viability of the lot. The vials are thawed, vortexed vigorously and the butyl rubber septum disinfected with 70% ethanol followed by flaming with a Bunsen burner. A sterile 24-well tissue culture plate is set up with 0.9 ml of sterile Tween–saline (0.05% Tween 80 in 0.85% NaCl solution) in each well. With a 1-ml sterile tuberculin syringe the suspension of bacilli is removed from the serum vial via the butyl stopper. A 0.5-ml sample of this suspension is plated onto a Trypticase soy or blood agar plate to check sterility. Into the first column of the 24-well plate is deposited 100 µl of sample into each row from each of the vials. With a P-200 Gilson Pipetman set at a 100 µl volume, and fitted with a barrier-type pipette tip, the samples are serially diluted through a series of 10-fold dilutions starting at column 1 and ending at column 6. Column 1 corresponds to a 1 : 10 dilution of the original vial as column 6 in the series corresponds to a 1 : 1 000 000 dilution. A volume of 100 µl is removed from each dilution well and plated onto quad-

rant-type Petri plates containing Middlebrook 7H11 agar. The plates are bagged in polypropylene zip-lock sandwich bags (4 plates per bag) and incubated in the dark at 37°C for 3 weeks. Colonies are then counted and the number of cfu of the mycobacterial frozen seed lot determined.

The number of cfu established for each seed culture lot is entered into the culture collection log, notebook or database. For each particular strain approximately 25–30 vials are retained as 'primary' seeds and not used except for subculture to establish more working stock. Repeated or continuous subculture of any strain of *M. tuberculosis* should be discouraged as this may cause physiological changes in the strain over time. If a strain needs to be subcultured or expanded it is advisable to start from one of the 'primary' seeds in the culture collection or, if available, from the original LJ slant. (*Note:* LJ slants can be maintained at 4°C and subcultured onto fresh LJ for maintenance of cultures.) The additional 70 vials or so from each initial subculture are then used as the working stock solutions.

Liquid Media

Proskauer and Beck liquid medium

Into 1 l of distilled water add each of the following ingredients in the order listed, making certain that each salt is dissolved before the next is added: KH_2PO_4 5.0 g; asparagine 5 g; $MgSO_4 \cdot 7\ H_2O$ 0.6 g; magnesium citrate 2.5 g; glycerol 20 ml; and Tween 80 0.5 ml (optional for dispersed cultures, 0.05% final concentration). The pH is then adjusted to 7.8 by the addition of 3–5 ml 40% NaOH. The medium is autoclaved at 121°C for 15 min on slow exhaust, after which the pH should be 7.4. Occasionally a precipitate forms after autoclaving; if this occurs, the medium should be allowed to cool and the precipitate removed by filtration. The medium is then autoclaved again at 121°C for 15 min (or can be sterile-filtered through a 0.2-µm filter unit or cartridge (Gelmann)).

Glycerol alanine salts

To prepare GAS, each of the following ingredients is dissolved in the following order into 990 ml distilled water: Bacto Casitone (pancreatic digest of casein; Difco) 0.3 g; ferric ammonium citrate 0.05 g; K_2HPO_4 4.0 g; citric acid (anhydrous) 2.0 g; L-alanine 1.0 g; $MgCl_2 \cdot 6H_2O$ 1.2 g; K_2SO_4 0.6 g; NH_4Cl 2.0 g; and glycerol 10 ml. The pH is adjusted to 6.6 by the addition of 1.8 ml 40% NaOH. The GAS medium is then autoclaved at 121°C for 15 min on slow exhaust.

Solid Media

Middlebrook 7H10 and 7H11 agar

Both 7H10 and 7H11 agar media (Difco) are routinely used for the cultivation and enumeration of *M. tuberculosis* by limiting dilution plating.

The 7H11 agar contains a pancreatic digest in the base, whereas 7H10 does not; this additive seems to enhance the growth of many strains of mycobacteria, and hence is the preferred medium in our laboratory. Both formulations, however, produce acceptable colony growth over a 3-week incubation at 37°C.

To prepare 1 l of 7H11 or 7H10, place the following ingredients into a 2-l Erlenmeyer flask: 7H11 (or 7H10) agar base 21 g; asparagine 1.0 g; glycerol 5.0 ml; and distilled water 900 ml. All the ingredients are thoroughly dissolved and the flask is capped with heavy aluminium foil, which is then secured into place with autoclave tape. The agar is then autoclaved for 15 min at 15 lb $inch^{-2}$ and 121°C on the slow exhaust cycle. Remove the flasks from the autoclave and check the colour of the agar. The agar should be emerald green; however, if it appears to be olive green or brown, discard the batch as it may have been 'overcooked' during autoclaving and the Malachite Green has degraded. (The breakdown of Malachite Green produces compounds that inhibit the growth of mycobacteria). After checking the colour of the agar, place the flask into a 57°C water bath and allow the agar to equilibrate for at least 45 min. While the agar is cooling, set up the following materials in a sterile medium preparation bench:

- One sterile 10-ml Cornwall syringe set to dispense 5.0-ml aliquots (if dispensing into X-plates; if using non-divided plates, disregard the Cornwall syringe).
- 60 No. 1009 X-plate Petri dishes for each litre of agar.
- One wire basket to support the 2-l flask.
- Sharpies to code plates as to possible drug additives.
- 100 ml oleic acid–dextrose complex (OADC) enrichment medium per litre of agar (described below).

When the agar has equilibrated to 57°C, remove the flask from the water bath and place it in the wire basket in the bench. Carefully remove the foil and aseptically pour 100 ml OADC enrichment medium into the litre of agar. Gently swirl the flask to mix the OADC throughout the agar. At this time, drugs such as TCH or INH can be added by filter sterilization. Carefully unwrap the Cornwall syringe from its foil envelope and aseptically place the tubing into the flask. Prime the syringe and proceed to dispense the agar into the Petri dishes with strict aseptic technique. Dispense 5.0 ml agar per quadrant per plate, or pour approximately 20 ml agar into a non-divided plate. After dispensing the medium, allow the plates to stand at room temperature for at least 2 h. This is sufficient time to allow the agar to solidify. Cover the plates with a dark plastic bag or aluminium foil to protect the medium from the light.

When the agar has solidified, put the plates into plastic storage trays and place these trays in a dry 37°C incubator for 24–48 h. This is a 'curing' step in which the excess moisture is removed from the agar and allows an opportunity for any contaminants to become apparent. After the incubation of the plates has been accomplished the plates are placed into ziplock polypropylene bags (5 plates per bag) and labelled with the type of agar, drugs, date and name. If the plates are not to be used immediately, they are stored at 4°C until use. Plates can be stored for up to 1 month; use after this time is not recommended.

Middlebrook oleic acid–dextrose complex

To prepare 4.0 l of OADC the following ingredients need to be dissolved in 3.8 l of distilled water in the order given: NaCl 32.4 g; bovine albumin fraction V 200 g; and dextrose (D-glucose) 80 g. When everything is in solution the pH of the mixture is adjusted to 7.0 with 4% NaOH.

To this mixture a sodium oleate solution is then added. This solution is prepared with the following ingredients: distilled water 120 ml; 6 M NaOH 2.4 ml; and oleic acid 2.4 ml. Once the sodium oleate solution has been prepared it is warmed in a 56°C water bath until the solution is clear. The complete OADC enrichment medium is then filter sterilized through 1.0 µm and then 0.2 µm cartridge filters (Gelman) using a peristaltic pump system. The medium is dispensed in a laminar flow tissue culture hood into sterile 100-ml bottles, which are capped, heated in a 56°C water bath for 1 h, and then incubated at 37°C overnight. The heating and incubation is then repeated the following day. After inspecting the OADC medium for contamination, the bottles are stored at 4°C for up to 2 months.

Adding drugs to media

Acriflavine

- Working concentration: 100 µg ml^{-1} (= 100 mg l^{-1}).
- Preparation: dissolve 100 mg Acriflavine in 10 ml sterile distilled water and filter sterilize. Add all 10 ml to 1 l agar.

Isoniazid

- Working concentration: 0.2 µg ml^{-1} (= 200 µg l^{-1} = 0.2 mg l^{-1}).
- Preparation: Dissolve 20 mg isoniazid (INH) in 10 ml sterile distilled water. Dilute this 1:10 in sterile distilled water to give a final concentration of 200 µg ml^{-1} and filter sterilize. Add 1 ml of the 200 µg ml^{-1} stock to 1 l of agar.

2-Thiophenecarboxylic acid hydrazide

- Working concentration: 1 µg ml^{-1} (= 1 mg l^{-1}).
- Preparation: Dissolve 10 mg 2-thiophenecarboxylic acid hydrazide (TCH) in 10 ml sterile distilled water and filter sterilize. Add 1 ml of this stock to 1 l of agar.

◆◆◆◆◆◆ PROPAGATION METHODS FOR THE ISOLATION OF IMMUNOLOGICALLY REACTIVE PROTEIN FRACTIONS

Equipment and Reagents

The growth of *M. tuberculosis* described in this section is for 500–1000 ml cultures, i.e. much higher volumes than for infecting inocula. Because of this, some growth conditions are different than those given above.

The equipment required includes a roller-bottle apparatus or platform shaker for gentle agitation of cultures and a class II biological safety cabinet. *M. tuberculosis* is a class III pathogen and its propagation must be performed in a certified BSL-3 laboratory (Centers for Disease Control, 1993). Several types of broth medium are available for the growth of *M. tuberculosis*; however, media containing supplemental proteins such as bovine serum albumin (BSA) or yeast extract should be avoided. We generally use GAS broth (see above). This medium allows for ample growth of the tubercle bacilli and is easily prepared.

Growth of *M. tuberculosis*

For initial growth of *M. tuberculosis* a 1-ml aliquot from frozen stock is inoculated on a Middlebrook 7H11 agar plate and incubated at 37°C for two to three weeks. Once colonies are visible a liberal inoculum of cells are scraped from the plate and placed in GAS broth (20 ml). This is allowed to incubate at 37°C with gentle shaking for two weeks. An aliquot (10 ml) of this seed culture is transferred to 100 ml of GAS broth. Following 2 weeks of incubation the entire 100 ml culture is used to inoculate 1 l or is split for 500 ml GAS broth cultures. These cultures are incubated in roller bottles or fernbach flasks with gentle agitation for 2 weeks. It is our experience that 2 weeks of incubation provides cultures that are in a mid- to late-logarithmic phase of growth (Sonnenberg and Belisle, 1997).

Harvesting Culture Filtrate Proteins and Preparation of Subcellular Fractions

Equipment and reagents

The separation of the cells and the culture supernatant is performed in a class II biological safety cabinet in a BSL-3 laboratory. The supernatant is harvested using 0.2-mm ZapCap filters (Schleicher & Schuell, Keene, NH, USA). Vacuum for this procedure is provided by a vacuum pump (maximum vacuum 26 in.Hg) with an in-line 0.2-mm filter (Gelman, Ann Arbor, MI, USA, catalogue No. 4251) placed between the ZapCap and the vacuum pump. Concentration of the filtrate is accomplished with an Amicon apparatus (Beverly, MA, USA). Harvesting of the cells requires a table-top centrifuge such as a Sorvall RT-6000, with sealed buckets for containment of potential aerosols. Lysis of the cells requires a French press or probe sonicator.

Breaking buffer

- 10 mM Tris-Cl, pH 7.4
- 150 mM NaCl
- 10 mM EDTA
- 100 mg ml^{-1} DNase

- 100 mg ml^{-1} RNase
- proteinase inhibitors.

Harvesting of CFPs

The bacilli in 500- or 1000-ml cultures are allowed to settle, and the supernatant is filtered through a 0.2-mm ZapCap filter into 4-l bottles. To minimize plugging of the filter it is important to use ZapCap with a prefilter and to decant a minimal amount of cells along with the supernatant. Sodium azide is added to the filtrate to a final concentration of 0.04% w/v, and this material is stored at 4°C. After filtration the culture supernatant is considered sterile. Nevertheless, before further use, a 1-ml aliquot of this filtrate is plated on Middlebrook 7H11 agar and incubated for 3 weeks to ensure the absence of viable bacilli. The filtrate is concentrated to approximately 2% of its original volume using an Amicon apparatus with a low protein binding, 10 kDa molecular weight cut-off membrane. This concentrate is dialysed extensively against 10 mM ammonium bicarbonate, and the protein concentration estimated by the BCA protein assay (Pierce, Rockford, IL, USA). The final culture filtrate proteins (CFP) preparation is aliquoted and stored at –70°C. Typically, 4–5 mg CFPs are obtained from a 1-l, 2-week culture of *M. tuberculosis*.

Isolation of Subcellular Fractions of *M. tuberculosis*

The cells from which the culture supernatant has been decanted are collected by centrifugation at 3000*g*. The cell pellet is washed with sterile H_2O and inactivated. To preserve the integrity of proteins γ-irradiation is recommended for killing of *M. tuberculosis*. A radiation dose of 2.4 Mrad renders all exposed bacilli non-viable, while not affecting the large majority of the enzymatic functions of the cells (Hutchinson and Pollard, 1961). Alternatively, *M. tuberculosis* cells can be killed by heating at 80°C for 1 h. This is best done in an autoclave with the capacity to perform low temperature isothermal cycles. However, heat killing is much more damaging to the integrity of the *M. tuberculosis* proteins. As with the CFP preparations, the lack of viable bacilli should be checked before cells are removed from containment facilities.

Several techniques for the generation of *M. tuberculosis* subcellular fractions have been reported (Hirschfield *et al.*, 1990; Lee *et al.*, 1992; Trias *et al.*, 1992; Wheeler *et al.*, 1993). Although some of these protocols may result in cleaner separation of subcellular fractions, we have found that the methods used by Hirschfield *et al.* (1990) and Lee *et al.* (1992) provide a rapid and simple means to obtain crude fractions of cell wall, membrane and cytosol. Lysis of *M. tuberculosis* cells is accomplished by suspending the cell pellet at a concentration of 2 g cells per millilitre of breaking buffer and passing this suspension through a French press at 1500 lb $inch^{-2}$, five to seven times. The lysate is diluted with one volume of the breaking buffer, and unbroken cells are removed by centrifugation at 3000*g* for 15 min. Cell-wall material is harvested from the supernatant of the low-

speed spin by centrifugation at 27 000*g* for 30 min. The cell-wall pellet is washed with 10 mM ammonium bicarbonate dialysed against the same, aliquoted and stored at –70°C. Supernatant from the 27 000*g* spin is further separated into cytosolic and membrane fractions by centrifugation at 100 000*g* for 1 h. The membrane pellet is washed, suspended in 10 mM ammonium bicarbonate and stored at –70°C. The cytosolic fraction is dialysed against 10 mM ammonium bicarbonate, aliquoted and stored. Extraction of proteins from the cell-wall or membrane fraction may be performed using a number of detergent or chaotropic agents that are easily removed prior to use of the proteins in immunological assays (Hjelemeland, 1990; Thomas and McNamee, 1990; Scopes, 1994).

◆◆◆◆◆◆ INFECTION PROTOCOLS

Intravenous Infection of Mice

The proper handling and restraining techniques for mice should be mastered prior to any attempt at intravenous inoculation with virulent strains of *M. tuberculosis*. The technician should seek proper training, which is provided by most institutional animal facilities, in order to ensure the safety of both the technician and the laboratory animal. Proper humane methods for handling laboratory mice should be learned by the technician and a certain degree of skill and confidence achieved prior to performing any work involving the use of *M. tuberculosis* in an animal model. The technician needs to be fully aware of the risks of accidental infection via a bite wound, scratch, accidental needle stick, or aerosol exposure when restraining and inoculating mice with *M. tuberculosis*. Because of this, it is extremely important that the technician develop a methodical approach when performing such techniques.

All infections of laboratory animals with *M. tuberculosis* should be done in an appropriate Animal Biosafety Level-3 laboratory (ABL-3) with the technician wearing appropriate facility clothing and personal protective equipment. Double, disposable surgical gloves should be worn when handling mice to prevent accidental exposure due to tearing of the outer glove (mice can bite through the outer glove, but rarely through both).

To infect mice intravenously the technician will require a preparation of *M. tuberculosis* from a stock of a known number of cfu per millilitre. The bacilli are thawed and carefully removed via a 1-ml tuberculin syringe fitted with a 26-gauge needle from the vial in a biological safety cabinet, and then diluted in normal saline or PBS to achieve the required cfu concentration per millilitre for intravenous challenge. When the mycobacterial suspension is dispensed from the tuberculin syringe into the dilution tube it is done in a manner in which the suspension is slowly dispensed down the inside wall of the tube. This is done in order to minimize the possibility of the creation of an aerosol from the needle. After dispensing the mycobacterial suspension, the syringe is carefully placed in an appropriate 'sharps' container for disposal.

The mycobacterial suspension is serially diluted and a sample plated on 7H11 agar to verify the concentration. Pipetting operations during the serial dilution process are done with extreme care in order to prevent aerosol generation. Any small spills or drops during the pipetting process should immediately be disinfected with 5% Lysol. All pipettes and other materials used to serially dilute the mycobacteria are placed in a pipette boat containing 5% Lysol solution. The biological containment hood is disinfected after use. The culture tubes, stock vials, and pipette boat are double bagged in autoclave bags and taped with heat-sensitive autoclave tape. These materials are then placed in a large autoclave pan located near the ABL-3 autoclave for subsequent sterilization.

In our laboratory, the standard intravenous challenge dose is 10^5 cfu per mouse. This dose was chosen because it is sublethal and allows the mouse to make a very strong cellular response. This is delivered intravenously in a volume of 0.2 ml from a working concentration of 5×10^5 cfu ml^{-1} suspended in normal saline or PBS. The injectate is loaded into a 1-ml tuberculin syringe fitted with a 26-gauge needle, with the needle bevel at the same orientation as the syringe graduations.

Prior to injecting mice, a full-face shield should be put on to prevent accidental exposure from injectate inadvertently spraying back during injection (a common, and scary event to the novice). Although a simple shield is probably sufficient, our common handling of drug-resistant isolates has prompted us to use a RACAL AC-3 (Racal Health and Safety) respirator which consists of a belt with a blower unit fitted with HEPA filters, which delivers sterile air to a helmet face shield. This unit provides clear visibility and does not fog up as traditional face respirators often do.

A cage of mice is then placed under a heat lamp in order to warm gently the mice and increase their venous circulation. It is essential that the worker pays close attention that the mice are warmed but not heat stressed while under this lamp. While the mice are warming a mouse restrainer device is set up. We use a metal-cone-type restrainer which bolts to the edge or the laboratory bench (Fig. 1). When the mice are sufficiently warm, a mouse is removed from the cage by gently grasping its tail. It is then placed into the restrainer, with the tail sliding through the slot in the top of the restrainer. While holding the tail, swab the area to be injected with a 70% ethanol soaked gauze sponge or swab. Allow the ethanol to air dry. Identify a lateral tail vein and, keeping the needle bevel up, gently insert the needle parallel to the vein 2–4 mm into the lumen.

Once you are sure you are in the vein, inject the *M. tuberculosis* suspension slowly. No bleb should be visible if the needle has been inserted properly into the lumen of the vein; if a bleb appears, indicating failure to locate the vein, additional attempts may be made proximally. It is desirable to make the first attempt at injection as close to the tip of the tail as possible. *Never* force the syringe if you miss the vein, as spray back will occur, or the needle hub may dislodge from the syringe or tail and cause a subsequent aerosol of *M. tuberculosis*. When finished, withdraw the needle slowly and apply pressure at the injection site, with gauze if necessary, to achieve haemostasis.

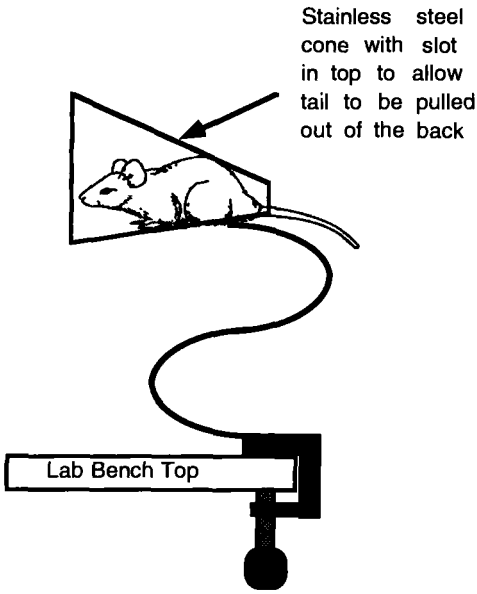

Figure 1. A simple mouse-restraint device for intravenous injections.

Aerosol Infection of Mice

Because the natural route of tuberculosis infection is the lung, the most precise animal models try to mimic this route. Intranasal or intratracheal inoculation of mice can give rise to pulmonary infection, but the most reproducible technique is to generate an aerosol that is inhaled by the animal. Aerosol devices are available in two main types: the sealed cabinet system pioneered by Middlebrook, and a newer device that fits over the nose of the animal. Whatever your choice, the device must be kept under stringent BL-3 conditions, due to the very high aerosol danger.

Our preference is the Middlebrook Airborne Infection apparatus, which has been used for this specific purpose for the past 30 years. This instrument is currently manufactured by Glas-Col, Inc. (Terre Haute, IN, USA), and has changed very little in configuration since the original design. The instrument consists of a large circular tank (aerosol chamber), which contains a circular basket/cage with five pie-shaped compartments in which animals are placed. Each of the compartments can accommodate as many as 25 mice, two 500-g guinea-pigs or one rabbit. The aerosol chamber has a heavy acrylic lid with two locking handles, which lock tightly against a heavy-duty rubber gasket. The lid also has two ultraviolet (UV) lamps on its underside, and these lamps are turned on during the decontamination cycle of instrument operation.

The front of the instrument consists of a control panel with four timers for the various cycles of operation; two air flow meters; two air control knobs; and an on/off switch for the exhaust-air incinerator. Also, on the front of the instrument are three tygon hoses with hose clamps for attach-

ing the glass venturi unit (nebulizer), which is fitted in a holding bracket and filled with the bacterial suspension. When the instrument is in operation, compressed air flows through the nebulizer and produces a very fine mist of the bacterial suspension, which is then carried by a larger volume of air flowing into the aerosol chamber. The airflow then exits the chamber through two HEPA filters and a superheated exhaust stack wherein the air is incinerated.

The timers on the control panel control the duration of the various cycles involved in the 'aerosol' process. The first timer controls a 'pre-heat' cycle which allows the incinerator to attain suitable temperature prior to nebulization. The second timer controls the time of nebulization and does not engage until the pre-heat cycle is completed. During nebulization the compressed air comes on and is routed through the venturi of the nebulizer to create the fine bacterial mist. The third cycle is the 'cloud decay' timer, and it is during this cycle that the aerosol chamber is purged with fresh air and the bacterial mist dissipated. The final timer is the 'UV cycle', in which the UV lamps are switched on and decontaminate the top surfaces of the basket.

Once all cycles are complete, the instrument is carefully examined to make sure the hoses and gaskets are still in place. The technician, wearing appropriate safety equipment (a RACAL safety helmet with HEPA filters is recommended (Orme and Collins, 1994)), then opens the chamber lid and removes the animals. The basket is carefully removed, wrapped in autoclave bags, and autoclaved to sterilize. The interior of the chamber is then disinfected with 5% Lysol followed by 70% ethanol. The glass venturi nebulizer is carefully removed and placed in a stainless-steel pan containing 5% Lysol, covered with a lid, wrapped in an autoclave bag, and sterilized by autoclaving. All disinfecting materials are likewise bagged and autoclaved.

◆◆◆◆◆◆ CULTURING AND INFECTING MACROPHAGES

Establishing Macrophage Cultures *In Vitro*

The establishment of primary cell cultures of murine bone marrow derived macrophages is an important *in vitro* technique, which is used extensively in our laboratory. These macrophages can be used for numerous types of studies ranging from macrophage responses to infection (NO, chemokine production for example), to evaluating the ability of drugs to prevent intracellular replication, to identifying protein targets of interferon (IFN) secreting T-cell subsets.

Bone marrow macrophage cultures are established by first euthanizing mice in accordance with the methods approved by your institutional animal care and use committee. We recommend placing mice in a large glass jar which has some pellets of dry ice on the bottom covered with gauze for carbon dioxide asphyxiation. Once the animals have been euthanized they are placed on a clean dissection board and saturated with 70%

ethanol. The skin at the midback is clipped with sterile scissors and then peeled back to expose the lower part of the body, including the hind legs. The knee is dislocated by holding the thigh with one hand and the shin with the other. Dislocation is accomplished by firmly pulling apart the knee joint. When this has been done, bend the knee back, holding the leg on the shin. Clip the muscle under the knee and bend the knee in the direction it normally would not bend. Pull down on the shin, and the femur will emerge through the muscle. Clip the femur from the hip, removing the excess muscle and place it in a tube containing ice-cold, sterile medium (see below). Clip the foot off, and place the sharp edge of the scissors between the tibia and fibula, and slide the scissors up the shin, cutting the muscle. Peel the tibia out of the muscle, and clip it from the knee, removing the excess muscle. The tibia is also placed in the tube containing ice-cold medium.

The tube containing the bones are then taken to the tissue-culture area of the laboratory and placed in a laminar-flow biological containment cabinet for the extraction of the bone marrow. The tube containing the bones and medium are carefully poured into a large sterile Petri dish. Sterile forceps and scissors are used to pick up the bones and clip the ends to expose the marrow. Using a 10-ml syringe filled with medium and fitted with a 26-gauge needle, rinse the bone marrow out of both ends of the bones into a 50-ml conical tube which contains 5-10 ml of medium. Rinse with about 4 ml of medium per bone, to ensure complete removal of all bone marrow cells.

A 10-ml pipette is then used to mix the marrow suspension gently up and down until all the clumps have been broken up. The bone marrow cells are then pelleted by centrifugation (1650 rpm, 7 min, 4°C). The supernatant is decanted and the cells resuspended (use 2 ml medium per animal harvested). Pass the cells through 100-mm sterile nylon mesh to break up cell clumps. Count the cells in 3% acetic acid/PBS (to lyse red cells), and then adjust the cell suspension to a concentration to 2×10^6 nucleated cells per millilitre of medium. Each mouse should provide 1×10^7 to 2×10^7 bone marrow cells. These are then plated onto Petri dishes, flasks, or well-type tissue culture plates, as shown in Table 1.

The bone marrow cell cultures are then placed in a 37°C incubator, which is supplemented with 95% humidified air containing 5–7% CO_2. The

Table 1. Bone marrow macrophage culture

Tissue culture system	Seed density (nucleated cells ml^{-1})	Volume required (ml)
75-cm TC-flasks	5.00×10^5	30.00
150-mm TC dishes	1.00×10^6	20.00
100-mm TC dishes	1.00×10^6	12.00
12-well TC plates	1.00×10^6	2.00
24-well TC plates	1.00×10^6	1.00
96-well TC plates	1.00×10^6	0.20

TC-flasks, tissue culture flasks that have a 75 cm^2 culture area; TC, tissue culture.

medium in the cultures is changed at 48 h after seeding and then again at day 4–5. The macrophages should have differentiated and formed a confluent monolayer by day 7–9. At this point the macrophages are mature and ready for use in experiments. If these bone marrow macrophages are to be used for infection with *M. tuberculosis*, the medium needs to be changed to antibiotic-free medium 48 h prior to the experiment.

Bone marrow medium

- Complete Dulbecco's modified Eagle's medium (DMEM) (Sigma No. D5530) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Sigma) and 10% L-929 conditioned medium
- *N*-[2-Hydroxyethylpiperazine-*N*'-2 ethanesulfonic acid] (HEPES) buffer (Sigma No. H0887)
- L-glutamine (Sigma No. G7513) minimum essential medium (MEM) non-essential amino acids (Sigma No. M7145)
- antibiotic/antimycotic (Sigma No. A9909) (omit for antibiotic-free bone marrow media).

L-929 conditioned medium

- L-929 cells from American type culture collection (ATCC) are grown up at 4.7×10^5 cells total in a 75-cm^2 T-flask with 55 ml DMEM (Sigma)
- 10% FCS (Intergen)

1. Allow cells to grow for 7 days or until confluent.
2. On day 7 collect the supernatant, filter through a 0.45-μm Nalgene filter and freeze at –20°C in 40-ml aliquots.

Infecting Macrophage Cultures

The *in vitro* infection of murine bone marrow derived macrophages is routinely used in our laboratory to evaluate the macrophage response to different clinical isolates, and also to determine the efficacy of various novel antibiotic compounds on the intracellular growth of *M. tuberculosis*.

In such studies, the macrophage monolayers are washed once with sterile phosphate buffered saline (PBS) and then supplemented with fresh antibiotic-free bone marrow macrophage medium, as described above. The cultures are allowed to incubate for 48 h prior to infection with *M. tuberculosis*. If the protocol involves cytokine treatment of the cells, these are added at this time.

The resulting macrophage monolayer contains approximately 1.0×10^7 cells after 8–10 days of incubation (24-well plate method). On the day of infection the medium is removed from the macrophage monolayer and immediately replaced with 200 μl antibiotic-free medium containing 1.0×10^6 cfu (5.0×10^6 cfu ml^{-1}) of *M. tuberculosis*. The plates are returned to the 37°C humidified air–CO_2 incubator for 4 h. After this incubation period the monolayers are washed gently four times with 1 ml sterile PBS to

remove any bacilli that were not ingested. (Great care should be taken not to generate aerosols when doing this procedure.)

The cells are now ready for further use. Production of oxygen or nitrogen radicals can be measured by colorimetric assays, and supernatants can be collected to measure the secretion of chemokines or cytokines. The cells can be lysed in RNAzol and processed for the presence of mRNA for molecules of interest. Alternatively, the bacteria can be allowed to grow, and this can be followed by plating lysates (distilled water plus 0.05% Tween 80, then diluted in PBS) and plotting the number of cfu versus time.

It has been claimed that such cultures can be taken out for as long as a month. Certainly the cultures can look good under phase-contrast microscopy for up to 2 weeks in our hands, but even then some detached macrophages are seen ('floaters'), which can make the bacterial numbers appear lower than they really are. For this reason we do not go further than 8 days for these assays.

A similar caveat regards using radioactive uracil as opposed to counting the cfu in macrophage cultures. In our hands, reduction in uracil uptake usually only reflects stasis, not cidal activity, so any claims that a reduction in uracil counts equals killing should be backed up by showing a reduction in cfu counts (Rhoades and Orme, 1997).

◆◆◆◆◆◆ IDENTIFYING KEY PROTEINS RECOGNIZED BY IMMUNE T CELLS

The bone marrow derived macrophage system can also be utilized in immunological assays involving antigen processing and presentation to T cells. In our laboratory we have had quite variable results based on cell-proliferation assays, and so, given our demonstration of protective T cells as cells that secrete IFN (Orme *et al.*, 1992; Cooper *et al.*, 1993), we have developed the following assay to detect the presence of these cells, and to obtain a picture of the proteins that they recognize. Details of these results are published elsewhere (Roberts *et al.*, 1995).

The system uses bone marrow derived macrophages to present antigen to T cells (or subsets thereof) harvested from syngeneic mice that have been intravenously infected with 10^5 cfu of *M. tuberculosis* 15 days earlier. (This is the timing of the peak response; other times can be chosen if one is interested in the kinetics of response to a given antigen (Orme and Collins, 1994).)

Tissue culture plates (96 well) are seeded with 2×10^5 bone marrow cells 7 days prior to analysis. Samples of individual proteins or protein fractions are dissolved in sterile pyrogen-free water to a concentration of 1 mg ml^{-1}. The medium in the bone marrow macrophage cultures is carefully removed and replaced with fresh medium. For pulsing macrophage cultures the stock protein samples must be diluted to a working concentration of 10 μg ml^{-1}. Ovalbumin is used as an irrelevant negative control (there is some background IFN secretion, usually in the picogram range). The macrophage cultures are then incubated overnight at 37°C and 6% CO_2 to allow processing and presentation of the test antigens.

The following day, infected mice are euthanized and spleen cell suspensions prepared. These are then incubated at 37°C in 6% CO_2 for 1 h in plastic flasks to allow macrophages to adhere. After incubation the plate is removed from the incubator and gently rocked/swirled to resuspend the non-adherent spleen cell population. The non-adherent cells are gently pipetted off into a sterile 50-ml conical tube and centrifuged at 200*g* for 7 min. The supernatant is gently decanted and the cell pellet is resuspended with 5 ml ACK lysing buffer (see below) per spleen in order to remove red blood cells from the non-adherent spleen cell population. The cells are incubated for 5 min at room temperature, with occasional shaking. (*Note:* ACK lysing buffer needs to be used at room temperature in order to work properly.) After the ACK incubation is complete, the tube is filled to 45 ml with tissue culture medium and again centrifuged for 7 min. This wash process is then repeated one additional time. After the last wash, the supernatant is decanted and the cell pellet is resuspended with monoclonal antibody supernatant preparations from clones TIB-210 (clone 2.43 anti-CD8.2) and TIB-183 (clone J11d.2 anti-B-cell, granulocyte, immature T-cell supernatant).

The exact working dilutions need to be determined for each lot of antibody supernatants. After incubation at 37°C for 30 min, Cederlane Low-Tox-M rabbit complement (1 : 16 final concentration) is added to the cell suspension and incubated for a further 1 h. The cells are then centrifuged at 200*g* for 7 min, and repeated two additional times. These cells should be an enriched population containing predominantly CD4 T cells, which can be checked by flow cytometric analysis.

The cells are then counted and resuspended 1×10^6 cells ml^{-1} in medium containing 20 units of interleukin-2 (IL-2) per millilitre to maintain T-cell viability. The T cells are then added in volumes of 0.1 ml to the wells containing the macrophages and incubated for 72 h at 37°C in 6% CO_2. The supernatants are then removed and assayed for IFN-γ by enzyme-linked immunosorbent assay (ELISA).

ACK lysing buffer

1. 0.15 M NH_4Cl (8.29 g); 1.0 mM $KHCO_3$ (1.0 g); 0.1 mM Na_2EDTA (37.2 mg).
2. Add 800 ml H_2O and adjust pH to 7.2–7.4 with 1 N HCl.
3. Add H_2O to 1.0 l.
4. Filter sterilize through 0.2-μm filter and store at room temperature.

Complete tissue culture medium

- Complete DMEM (Sigma No. D5530) supplemented with 10% heat-inactivated FCS (Sigma)
- HEPES buffer (Sigma No. H0887)
- L-glutamine (Sigma No. G7513) MEM non-essential amino acids (Sigma No. M7145)
- antibiotic/antimycotic (Sigma No. A9909).

◆◆◆◆◆ FOLLOWING THE COURSE OF INFECTION

Following the course of the tuberculosis infection by plating tissues and counting bacterial colonies can give you valuable information about the kinetics of expression of the host response (Orme, 1995). You should also be aware, however, that the assay is rather crude, with a counting error of about 20%. Because of this variance, one cannot read too much into small changes in numbers; as an example, late stages of the infection often appear to be 'chronic', i.e. flat-line. The infection may truly be chronic, but could also be increasing and waning over a small range; there simply is no way to tell.

One can also use bacterial counts to see the effects of a treatment, such as chemotherapy, vaccination or gene disruption of the mouse. Again, given the biological variation, a statistical difference is not seen unless the mean values differ by about 0.5–0.7 log.

Simple power statistics show that four mice per group is the minimum number that should be used *in vivo*. Thus, to follow the course of infection, one should harvest four mice at each time point. After a while, one gets an intuitive sense of which time points to choose; even so, it is always sensible to have a few spare mice just in case.

We place organs in individual industrial-strength homogenizing tubes with a very tight pestle (keep on dry ice before using so that the pestle does not expand) and grind for 30–40 s. Do not worry about the high shearing forces; these are needed to disrupt all the cells and disperse the bacteria. Then make serial dilutions and plate 100 µl of each dilution on quadrants of Petri dishes containing 7H11 agar. Keep the homogenates on ice at all times.

Incubate the plates in a 37°C cabinet containing a tray of water to keep the air humid and prevent the plates drying out. Resist the temptation to count colonies when they are still small (you will undercount). Count the quadrant in which there are between 10 and 100 colonies, but also make sure that the other quadrants correlate; i.e. if you have about 40 colonies, there should be about 400 on the one above and about 3–5 on the next dilution down. Then apply dilution factors and the original homogenate volume to calculate the total bacteria per organ.

If you see no colonies on 0 or –1 dilutions of the liver tissues, but there are some at higher dilutions, do not worry. The liver contains enzymes that inhibit bacterial growth; as these are diluted out the colonies start to grow.

A further 'quality control' is to do a day-1 count; i.e. 24 h after initiating infection. The numbers you obtain here can tell you if the infectious dose required was obtained. After intravenous infection, expect about a 90%/10%/2% distribution in uptake in the liver, spleen, and lungs. Look especially at the lung counts; if the uptake was 25–50% in the lungs instead of a few per cent, then your inoculum was clumped (and the rafts got stuck in the lungs).

The sensitivity of the assay is about 1.7 log; below this we usually designate the result as not detected (ND). In fact, low numbers or zero colonies can sometimes be a problem; if you are using a computer pro-

gram, do not incorporate a zero, as this will undercount your values. In the same vein, be careful about very high values; a common mistake made by beginners is not to change pipettes between dilutions. As a result one 'carries over' bacteria from one dilution to the next, which can escalate the count by as much as 3–4 log. Use common sense; if the bacterial load in wet weight is larger than the weight of the mouse, you did something wrong!

◆◆◆◆◆◆ DELAYED-TYPE HYPERSENSITIVITY AND NON-SPECIFIC RESISTANCE

The delayed-type hypersensitivity (DTH) reaction is the immunological reaction underlying the clinical skin test for tuberculosis. After injection of 'tuberculin' (purified protein derivative (PPD) of tuberculin), dermal Langerhans cells, dendritic cells or tissue macrophages ingest the PPD antigens and present them to recirculating T cells. These cells then generate signals (chemokines) that attract the egress from the blood of monocytes, leading to swelling of the local tissues as they fill with cells. Because the ability to mount a DTH reaction to tuberculin usually arises concomitantly with the expression of protective immunity, then this reactivity is taken to indicate exposure to the infection.

In the mouse the DTH reaction to tuberculin is very small, with a highly immune animal giving a reaction of about 0.5 mm. The use of precision dial-gauge calipers to measure these small changes is recommended, and if possible the experiment should be done blind by an 'uninterested' colleague.

To elicit a DTH reaction, inject a hind foot pad with 30 µl of 5–10 µg PPD (or test protein). Use a 30-gauge needle and inject between the 'thumb' and 'forefinger' of the foot. Inject the other foot pad with the same volume of diluent only.

Measure the size of the two feet 24 and 48 h later. Support the foot on the bottom crown of the caliper and bring the upper crown down onto the foot. If the toes begin to curl up you are starting to compress the foot. The readings should be about 0.18–0.21 mm for control feet and about 0.25 mm for the test response in 25-g mice.

A second element of the host response that can be measured *in vivo* is the development of non-specific resistance. This refers to the activation of (mostly uninfected) macrophages that have entered the site of infection and have become exposed to IFN-γ and other cytokines. This is an important parameter to measure under certain experimental conditions; for example, testing the effects of an immunomodulator. Earlier in the history of the field, great emphasis was made on certain mycobacterial materials that appeared to vaccinate mice against *M. tuberculosis* challenge; in fact, the whole thing was due to increased non-specific resistance caused by the adjuvant properties of the materials. If you think resistance is specific, rather than non-specific, then you must formally show that this resistance can be transferred by T cells (Orme, 1988).

The classical way to measure non-specific resistance is to inject the mycobacteria-infected mouse with a lethal dose of *Listeria monocytogenes* and then plate the liver and spleen 24 h later to see how much of the *Listeria* inoculum has been destroyed. To do this, infect groups of mice intravenously with 10^5 *L. monocytogenes* strain EDG. Harvest organs 24 h later and plate serial dilutions on trypticase–soy agar. Incubate for 18 h in humidified air and count the colonies. In a highly activated mouse expect to see about a 3–4 log decrease in bacterial load versus control (naive) mice.

◆◆◆◆◆◆ MEASURING THE CHEMOKINE/CYTOKINE RESPONSE *IN VIVO*

Over the past several years the importance of cytokines in the initiation, development and control of the immune response has become well established. With this fact in mind, we have been defining the role these potent molecules play in the immune response to infection by *M. tuberculosis*.

A primary goal in our laboratory has been to detect the presence of these cytokines during the experimental infection of the mouse. As a first broad measure we detect cytokine-specific mRNA in the infected tissue by reverse transcribing total RNA and amplifying specific sequences using cytokine-specific primers (Svetic *et al.*, 1991; Rhoades *et al.*, 1995; Cooper *et al.*, 1995a,b). This provides a glimpse of the events occurring within the tissue as the infection progresses.

The detection of cytokine-specific mRNA is quite straightforward for lung tissue, as this organ has low endogenous levels of cytokine expression, which allows easy recognition of a positive signal. We can therefore use this technique to glean a substantial amount of information about the cytokines that are induced as a consequence of an aerosol infection. It is difficult, however, to determine the actual amount of protein within the tissue, as the levels are generally too low to be detected by an ELISA. To identify which cytokines are being generated as a result of infection, we are therefore obliged to culture cells *ex vivo*. This allows the protein to accumulate to levels detectable by ELISA. The most straightforward tissue from which to extract cells for this purpose is the spleen from an intravenously infected mouse. This organ contains many of the cells of the immune system and is also a primary target organ following this route of infection.

Cytokines are not only generated in an innate fashion directly in response to infection, but are also produced by the antigen-specific cells of the acquired immune response. These cells are of primary importance in our studies, as they are the cells that need to be induced in order to control infection fully. To determine cytokine secretion by these cells, splenic (intravenous infection) or thoracic (aerosol infection) lymphocytes are cultured in the presence of mycobacterial antigens, and the supernatants analysed by ELISA for cytokines.

For these studies we take either lung (aerosol infection) or liver (intravenous infection) samples from each of four mice sacrificed at each time point. Only a small piece of tissue is required, and tissue for histology and for bacterial counts can be used from the same animals. Usually the corresponding lobe of tissue is taken from each animal; however, in more extensive studies two lobes from each animal may be processed in order to maximize confidence in the results. Although this approach can result in 80 samples per experiment, this is still a manageable number using the 96-well plate approach.

Immediately following sacrifice the tissues to be studied should be removed from the animal and placed in Ultraspec (a preparatory solution containing chaotropic agents and RNase inhibitors from Biotecx TX). The sample should then be homogenized using a metal Polytron homogenizer (if this tissue contains virulent mycobacteria then this should be done in a glove box) and immediately frozen in liquid nitrogen. The frozen samples can be stored at –70°C until all samples from the experiment have been collected. The Ultraspec can protect the RNA for a few hours or more at room temperature, and normal precautions for handling RNA should be observed throughout the extraction procedure (Sambrook *et al.*, 1989).

The RNA can be extracted from the homogenized tissue into chloroform/Ultraspec (1:5 v/v); the RNA is then decanted from the aqueous phase into isopropanol, and pelleted by centrifugation. The pellet should be resuspended in RNase-free distilled water (Sambrook *et al.*, 1989).

Once the RNA has been dissolved in water, the optical density (OD) at 260 nm should be read to determine the amount of nucleic acid in the preparation. To determine the amount of contaminating protein still in the preparation, the OD of the sample at 280 nm should also be read. If the ratio of the 260-nm reading to the 280-nm reading is around 2.00, then the nucleic acid is clean and the PCR reaction should be able to proceed unhindered. If the ratio is lower than 1.70 the PCR may well be inhibited to varying degrees depending on the contaminant. The samples should therefore be reprecipitated if the ratio is less than 1.70. It is possible to proceed with RNA of poor purity, such as in the case of rare samples, but expect your results not to be as clear-cut.

Although the nucleic acid may be very pure it may also have been degraded through poor handling. One can check the integrity of the RNA by gel electrophoresis, but this consumes sample and will not identify any contamination problems. It is thus advisable to proceed with the reverse transcription and then amplify a known housekeeping gene by PCR. If the housekeeping gene (we use HPRT) can be amplified equally well from each sample, then the RNA is known to be intact.

The reverse transcription is undertaken with 1 μg RNA (this was determined by measuring the OD; a value of 1.00 at 260 nm is equivalent to 40 μg RNA). RNA in a volume of 11 μl of water is added to the following mixture: 2.5 μl of a 2.5 mM mixture of all four deoxyribonucleotides; 5 μl enzyme buffer; 2 μl 0.1 M dithiothreitol; and 2 μl random hexamer oligonucleotides. This mixture can be made in bulk prior to adding samples. The sample is

added and the mixture is added to the wells of a 96-well plate specific for a thermal cycler, and oil is placed on top. The nucleic acid is treated for 5 min at 70°C and then 1 µl murine Moloney virus reverse transcriptase is bubbled into the mixture. The sample is incubated for 1 h at 37°C and the enzyme is killed by incubation at 95°C for 5 min. The resultant 25 µl of cDNA is then added to 200 µl pure water, and the sample is ready to undergo the PCR. The cDNA is diluted so that several cytokines can be detected from the same reverse transcription product.

As noted above, the first PCR is for the housekeeping gene HPRT. If some of the samples do not give an equal signal for HPRT, then the original RNA is reprecipitated and reverse transcribed again. We do not proceed with the cytokine PCR until all the HPRT signals are within two-fold of each other. Once the cDNA has been made, the cytokine-specific reactions should be performed quickly. In addition, it is advisable to re-measure the HPRT at the end of the cytokine reactions in order to confirm the quality of the RNA. It is possible to perform about 20 reactions from one reverse transcription.

The diluted cDNA (10 µl) is added to the following mixture: 4 µl 2.5 mM dNTPs; 5 µl PCR buffer (containing 15 mM magnesium chloride); 4 µl of a 0.2 µM mixture of sense and antisense primers; and 26.8 µl RNase-free water. Last, add 0.2 µl *Taq* polymerase (5 units μl^{-1}). Seal the wells with oil and place in a thermal cycler. When the appropriate number of heat/cool cycles have been run, heat again to 72°C for 7 min prior to analysis.

The only difference between cytokine programmes lies in the number of cycles undergone. The smaller the amount of specific RNA, the more cycles are needed to amplify the signal to give a good final chemiluminescent response. To determine this, an RNA sample known to contain a large amount of the specific cytokine mRNA is reverse transcribed, and the cDNA double diluted eight times. The cDNA is then amplified for a variety of cycle numbers and the signal quantified. The cycle number that gives the broadest range of detectable signal is then deemed appropriate for future experiments. It is necessary to determine cycle numbers for each new primer pair, for a new source of *Taq*, and for each individual thermal cycler. In addition, the cycle number is sometimes not the same for RNA derived from purified cells when compared to whole tissue derived RNA.

The amplicon can be run on a 1% agarose horizontal gel (a 20 × 20 cm gel box with 5 × 20 toothed combs can handle 100 samples) and blotted via capillary action onto nylon membrane (Hybond N, Amersham). The nylon membrane is then exposed to 1200 kJ UV light, which binds the amplicon to the membrane.

The specific amplicon is detected on the membrane using labelled sequence-specific probes, freshly made each time. The membrane is incubated at 42°C overnight and the blot is washed at 45°C to remove probe that may have bound non-specifically. The membrane is then exposed to a fluorescein specific antibody labelled with horseradish peroxidase (HRP), and then to an agent which emits light photons in the presence of HRP. This is detected using chemiluminescent film (Hyperfilm, Amersham).

The size of the band seen on the film is related to the amount of specific probe on the membrane, and thus to the amount of cytokine-specific sequence in the amplicon. The size of the band can be analysed using a flat-bed scanner and imaging software (such as the NIH Image software available via Shareware).

The size of each band for each mouse within an experimental group is divided by the size of the band for each of four control uninfected animals. This generates a series of values for the fold increase in signal in the experimental groups compared to the control group. The mean and standard deviation of these values is usually plotted against time. The HPRT graph should be flat at a value of 1, whilst 10- to 100-fold increases can be seen in the expression of specific cytokines responding to the infection.

Key aspects to the credibility of this approach are the use of at least four mice per experimental group, the careful preparation of the cDNA so that the HPRT values are equivalent, and the use of the optimal cycle number for each cytokine. One major advantage to this technique is that old RNA samples stored in the freezer can be analysed for any newly discovered cytokine as soon as the sequence has been published.

ELISA Analysis of Cytokines following *Ex Vivo* Culture of Organs

As noted above, the spleen is the most suitable organ for the direct analysis of an organ's response to infection. Following intravenous infection, the spleens of four mice are removed and then pushed gently through nylon spleen screens with the plunger of a 10-ml syringe. The resultant single-cell suspension contains a wide variety of cells, including both mononuclear and polymorphonuclear cells. The cell suspension should be kept on ice to avoid loss of any macrophages, which will stick to warm plastic. The red blood cells are then lysed and the remaining cells counted and plated at 1×10^6 per well in 96-well plates. Cells should be cultured alone, in the presence of 25 $\mu g\ ml^{-1}$ antigen or 2 $\mu g\ ml^{-1}$ of the mitogen concanavalin A. The culture can be left for 5 days and then analysed by ELISA for IL-6, IL-10, IFN-γ, TNFα, IL-12 p40 and p70. If the levels of IL-2 and IL-4 are required, then cultures should be left for only 24 h, as these growth factors will be consumed by proliferating cells. Pharmingen produces matched antibody pairs and standards for many cytokines. We use a straightforward sandwich ELISA procedure as follows:

1. Place primary antibody at 1 $\mu g\ ml^{-1}$ in coating buffer, 4°C overnight in Immulon 2 plates from Dynatech.
2. Flick out primary antibody and block the non-specific protein binding sites using 3% BSA in PBS (0.1% Tween, PBST) for 2 h at room temperature.
3. Wash the plate four times with PBST, add the sample and standard diluted in cell culture medium (if samples are from infected animals, this and the following steps should be done under BL3 conditions). Leave at 4°C overnight.

4. Wash the plate four times as above. Add biotinylated secondary antibody at 1 μg ml^{-1} in the BSA blocking solution and leave for 45 min at room temperature.
5. Wash the plate four times. Add avidin–peroxidase in working buffer and allow to develop.
6. Read the plate at 405 nm.

This procedure can also be used on the thoracic lymph nodes of aerogenically infected mice. However, these organs may need to be pooled together to generate enough cells for analysis.

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4.2 The Leishmaniasis Model

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INTRODUCTION

The study of experimental infection with the intracellular protozoan parasite *Leishmania major* has not only contributed significantly to our understanding of this fascinating host–parasite relationship, but also to many basic immunological phenomena. Much has been learned about the interaction of antigen-specific T cells and antigen-presenting cells, about cytokine and T-cell subset regulation, and co-stimulatory requirements. The immune response to experimental *L. major* infection in inbred strains of mice is the paradigm for polarized T-helper (Th) cell differentiation: in this model system, a Th1 response characterized by interleukin-2 (IL-2) and γ-interferon (IFN-γ) secretion leads to self-curing disease, whereas a Th2 response (IL-4, IL-10) leads to non-healing disease. Numerous manipulations, including the injection of cytokines and of neutralizing anti-cytokine antibodies, cytokine transgene expression, and more recently studies in cytokine and cytokine receptor gene knock-out mice, have all provided intriguing new pieces to the as yet incomplete mosaic of our understanding of the immune response.

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The leishmaniases are a group of parasitic diseases in humans caused by more than 20 different *Leishmania* species. They affect 88 countries world-wide and the disease incidence is still spreading (World Health Organization, 1995). The increase can partly be attributed to rapid economic development and the establishment of new settlements and construction sites in forest areas of the Amazon and other regions where the disease is endemic (PAHO, 1994). Early diagnosis is difficult, and treatment of leishmaniasis is costly and requires daily injections for weeks. Urbanization of the mostly rural diseases and the growing number of AIDS patients developing leishmaniasis as a secondary infection are becoming increasingly serious problems, especially as there is still no defined and efficient vaccine available for use in humans (Modabber, 1996).

◆◆◆◆◆◆ THE DISEASE AND THE EXPERIMENTAL LEISHMANIASIS MODEL

In humans, leishmanial infections range from self-healing cutaneous lesions to severe, non-healing disseminated cutaneous or visceral infections. To a large extent the clinical manifestations of the disease reflect the efficiency of the host's immune response to the parasite (Turk and Bryceson, 1971). Leishmaniasis, like leprosy, is considered to be a spectrum of diseases, because of the range of clinical forms in which it can be presented. Many of the clinical manifestations of human leishmaniasis can be mimicked in inbred strains of mice. Mice infected with different species of *Leishmania* have been used as models for human leishmaniasis and are very valuable in deciphering the immunological and genetic parameters associated with these diseases.

In this chapter we shall not use the frequently used terms 'resistant' and 'susceptible' to refer to mouse strains that are able or unable to heal experimental infections with *L. major*, for the simple reason that all strains of mice thus far tested can be infected with *L. major*, i.e. they do not resist infection; most develop small transient lesions at the site of infection, resolve them spontaneously within a few weeks, and remain solidly immune to reinfection thereafter. Of critical importance is the ability of these diverse mouse strains to heal such experimental infections. *L. major* infections in inbred strains of mice provide models for both healing and non-healing cutaneous leishmaniasis. The majority of inbred strains eventually heal cutaneous infections and only a very few strains of mice such as BALB/c, DBA/2 (Behin *et al.*, 1979; Handman *et al.*, 1979) and SWR (Nabors and Farrell, 1994b) develop severe, non-healing disease which, depending on parasite strain and dose, may lead to the death of the experimentally infected animal. It should be pointed out that the simplistic notion that non-healer mice die is an unfortunate distortion of the earlier categories proposed by Preston and Dumonde (1976), who characterized three clinical patterns of cutaneous leishmaniasis: self-healing, localized non-healing, and generalized non-healing. It is of prime importance to compare infections using the same dose of the same strain of the same

species of *Leishmania*: for example, low-dose infection of the paradigm non-healer BALB/c strain leads to healing and the development of protective immunity, which never happens with the standard infectious dose of 1×10^6 to 2×10^6 promastigotes. Virtually the entire range of clinical manifestations can be reproduced in a single mouse strain with one strain of *L. major*, depending on the syringe-injected infectious dose, leading to different outcomes depending on both the mouse strain, the parasite species and strain, the site of infection, and the number of parasites injected (Turk and Bryceson, 1971; Preston and Dumonde, 1976; Behin *et al.*, 1979; Handman *et al.*, 1979; Doherty and Coffmann, 1996).

Our understanding of the mechanisms resulting in this spectrum of clinical manifestations is based primarily on results obtained with the well-established mouse model for cutaneous leishmaniasis using *L. major*. However, contradictory results exist in experimental leishmaniasis. Currently it is very difficult to compare experimental results from different laboratories and the WHO designation for the *Leishmania* strain used (World Health Organisation, 1982) is not always indicated. Parasite culture conditions are not standardized and there are numerous differences in the maintenance and handling of the parasites: different strains of *L. major* with different virulence characteristics are used, they are cultured in different media, kept for different lengths of time in culture, the number of infective promastigotes in an infectious inoculum is hard to compare, and different routes of infection are used. In this chapter, we focus on the parasites and on the conditions relevant to initiation of successful infections. Specifically, the maintenance of virulent parasites, the preparation of metacyclic parasites used to initiate infection, the route of infection, and the determination of the disease progression by quantitating the burden of *L. major* parasites in various tissues will be described in detail.

The Parasite: Taxonomy and Life Cycle

The kinetoplastid protozoan parasites *Leishmania* belong to the family Trypanosomatidae, and share many common morphological features. *Leishmania* are unicellular organisms that possess a single anterior flagellum in the promastigote stage and are characterized by the kinetoplast, a structure unique to the order which is composed of many copies of concatenated mitochondrial DNA contained within a single tubular mitochondrion (Mendoza-Leon *et al.*, 1996).

Order:	Kinetoplastida
Family:	Trypanosomatidae
Genus:	*Leishmania*
Species:	*L. major*, etc.

Leishmania reproduce principally asexually by binary fission. Their life cycle includes an extracellular flagellated promastigote form in the invertebrate sandfly vector and an obligate intracellular form in mammalian macrophages, termed amastigotes. The promastigotes undergo a differentiation process of 'infectivity maturation' in the sandfly (Sacks and

Perkins, 1984). The sequential development of *Leishmania* promastigotes from a non-infective to an infective metacyclic stage is growth-cycle dependent and the infective stage is restricted to non-dividing organisms. The promastigotes and amastigotes represent the two morphologically and developmentally most extreme forms occurring in the vector and macrophage, respectively. The ovoid amastigote, and the motile, slender and flagellated promastigote live in distinct hydrolytic environments in the insect's digestive tract (midgut, neutral to alkaline pH) and the macrophage's phagolysosome (acid pH). The morphology of the flagellum is very distinct in these two forms: promastigotes have a flagellum that emerges from the anterior end of the body, whereas amastigotes possess a truncated flagellum that does not extend beyond the flagellar pocket (Mendoza-Leon *et al.*, 1996). Promastigotes found in the vector midgut differentiate from a non-infective to an infective stage in the proboscis (Sacks and Perkins, 1984) in a process termed metacyclogenesis (Da Silva and Sacks, 1987). In contrast to procyclics (in the midgut in the sandfly or in log phase culture *in vitro*), the kinetoplast and the nucleus are in close proximity in metacyclic promastigotes. The life cycle is completed when a sandfly bites an infected host and takes up promastigotes in the bloodmeal. A similar differentiation from non-infective into infective promastigotes occurs with lower efficiency in promastigote culture *in vitro*.

Leishmania avoid destruction by immune defence mechanisms of the host by expressing stage-specific molecules (Turco and Descoteaux, 1992; McConville and Ferguson, 1993). The dominant cell surface molecule of the promastigote is lipophosphoglycan (LPG), a glycophosphatidylinositol (GPI)-anchored carbohydrate polymer, consisting in its simplest form of repeating phosphorylated mannose–galactose disaccharides. This molecule, particularly the repeating moiety, is essential for the interaction of the promastigote with both the insect vector and the mammalian host. LPG is required for the establishment of infection inside the macrophage (Handman *et al.*, 1986; McNeely and Turco, 1990). During metacyclogenesis, the LPG of *L. major* undergoes changes in the expression and composition of the phosphorylated saccharide units (Sacks and Da Silva, 1987). The type and amount of LPG expressed appears to be responsible for the stage-specific attachment of the parasites. The several million copies of LPG at the surface of the promastigote form a dense glycocalyx, which may provide a physical protection barrier against defense mechanisms of the host, i.e. complement (Puentes *et al.*, 1988, 1990). LPG and other molecules including polymeric acid phosphatase (Ilg *et al.*, 1991) and a unique phosphoglycan (Ilg *et al.*, 1994) are released or secreted by promastigotes of many, but not all species of *Leishmania in vitro* and presumably *in vivo*. LPG plays multiple roles in the infectious cycle of *Leishmania* (Turco and Descoteaux, 1992; McConville and Ferguson, 1993). It is a multifunctional virulence determinant, and is involved in the growth of *Leishmania* in the sandflies, in the complement resistance of the parasites after infection, in attachment to macrophages, and in the establishment of successful parasitism in the mammalian macrophage (Beverley and Turco, 1995). Interestingly, it has been associated with both protection and exacerbation of disease (Turco and Descoteaux, 1992; Bogdan *et al.*, 1993).

The novel 63-kDa surface metalloproteinase leishmanolysin is expressed by promastigotes of *L. major*, but not by amastigotes (Schneider *et al.*, 1992). Leishmanolysin (EC 3.4.24.36; promastigote surface proteinase (PSP); gp63) may represent as much as 2% of the total promastigote protein and is thought to protect the parasite from complement-mediated lysis (Puentes *et al.*, 1990). Although its role in the infection of the mammal remains controversial (Bouvier *et al.*, 1995), the major surface antigen has received much attention as a vaccine candidate (Connell *et al.*, 1993). However, its inactivity at phagolysosomal pH (Bouvier *et al.*, 1990) suggests that it functions in the neutral-to-alkaline environment of the sandfly vector midgut.

The Experimental Infection

The differentiation stage of the promastigotes is responsible for very distinct effects in their mammalian hosts: natural and experimental infections are initiated by the infective stage of the parasites, the metacyclic promastigotes. Procyclic/log phase promastigotes are not or only poorly able to initiate an infection. This striking difference in infectivity during their life-cycle stage is one of the major reasons to pay attention to the developmental phase and to use methods that allow the separation of infective from non-infective promastigotes.

The two stages of the promastigote differ not only in infectivity, but also in their ability to interact with receptors on the surface of macrophages and to elicit a respiratory burst. Stage-specific expression of different cell-surface molecules might influence the ability of the *L. major* promastigote to establish itself intracellularly. Infective, metacyclic promastigotes bind to complement receptor 1 (CR1) on macrophages and enter these cells 'silently'; i.e. they do not elicit a strong respiratory burst, which clearly favours the survival of the invading parasite (Mosser and Edelson, 1987; Da Silva *et al.*, 1989). In contrast, procyclic promastigotes in the logarithmic growth phase bind to CR3 and elicit a high-level microbicidal respiratory burst and associated generation of toxic oxygen metabolites such as superoxide anion, hydrogen peroxide and nitric oxide.

As mentioned before, *Leishmania* express stage-specific molecules such as LPG, which are essential for host–parasite interactions. The repeating disaccharide units of LPG are required for promastigotes to establish infection and survive inside macrophages (Handman *et al.*, 1986; Beverley and Turco, 1995). Glycoconjugates of *Leishmania* LPG have been shown to have a regulatory effect on the induction of the inducible nitric oxide synthase (iNOS) (Proudfoot *et al.*, 1996), which catalyses the synthesis of high concentrations of nitric oxide (NO) from L-arginine and molecular oxygen. NO is involved in the killing of a range of micro-organisms (James and Hibbs, 1990; Liew and Cox, 1991) and *L. major* represents one well-characterized example (Liew *et al.*, 1990; Mauel *et al.*, 1991b; Wei *et al.*, 1995). The healing of *L. major* infection is clearly dependent upon the ability of macrophages to destroy the intracellular parasites (Behin *et al.*, 1979; Nacy *et al.*, 1983). Parasite killing by macrophages occurs after specific activation of the iNOS in macrophages by cytokines (Bogdan *et al.*, 1990, 1993).

Macrophages activated as a result of the specific immune response of the host are the decisive effector cells (Liew *et al.*, 1990; Mauel *et al.*, 1991b).

Immune Response to *Leishmania* Infection

It is generally accepted that the nature of the T-cell response is one of the crucial factors controlling mouse and human leishmaniasis, and one major advance in our understanding of the immune system was the discovery that Th cells can differentiate into functionally distinct T-cell subsets (Mosmann *et al.*, 1986), which alter and direct immune responses. Indeed, infection of mice with *L. major* is widely used as a model to study the differential development of $CD4^+$ Th cell subsets (Th1/Th2) *in vivo* (Scott *et al.*, 1989; Liew, 1989; Liew and O'Donnel, 1993; Pearce and Reiner, 1995; Reiner and Locksley, 1995). Polarized Th1 or Th2 responses are associated with healing or progressive disease in strains of mice genetically able to heal their infections or not, respectively. The inability to heal is correlated with the expansion of IL-4-producing Th2 cells, whereas the expansion of IFN-γ producing Th1 cells results in control of infection (Heinzel *et al.*, 1989; Scott, 1989; Scott *et al.*,1989; Heinzel *et al.*, 1991; Wang *et al.*, 1994; Reiner and Locksley, 1995). Cytokines released by one Th cell subset cross-regulate the development of the other subset: Th2 cells suppress Th1 cells by secreting IL-4, while IFN-γ inhibits Th2 cell expansion (Gajewski and Fitch, 1988; Mosmann and Coffman, 1989). Currently, there are no generally accepted cell-surface markers to distinguish different Th cell subsets. However, based on the cytokine pattern that these subsets secrete, it is possible functionally to identify at least three stable, mature types of Th cells: Th0, Th1 and Th2 . Precursor $CD4^+$ T cells leave the thymus as naive cells and, to express one of the functional Th phenotypes, they must be activated to differentiate into mature effector cells that secrete distinct cytokines. Although Th1 and Th2 cells contribute to the composition of the cytokine milieu in the infected host, $CD8^+$ T cells secrete similar arrays of cytokines and can also be subdivided into discrete cytokine-secreting subpopulations (Erard *et al.*, 1993; Sad *et al.*, 1995). The cytokines secreted by γδ T cells (Ferrick *et al.*, 1995), and by natural killer (NK) cells, mast cells and $NK1.1^+$ T cells also contribute to this milieu.

While cell-mediated immunity undoubtedly represents the major effector mechanism of protective immune responses, the role of humoral responses is not as clear. Cytokines control the type of immunoglobulin (Ig) subclasses expressed by B-cells. Th2 cells are largely responsible for providing B-cell help and are indispensable for IgE isotype switching (Mosmann and Coffman, 1989; Coffman *et al.*, 1993). Th1 cells, however, are not devoid of the capacity to help B cells. They mainly help for the production of antibodies of the IgG2a isotypes, but are unable to induce isotype switching to IgE. Therefore, the nature of the Th-cell response will strongly influence the Ig isotypes present. Analysis of IgG isotypes in patients with different manifestations of American cutaneous leishmaniasis confirmed a relationship between the Ig isotype in the serum and the form of disease of the patients (Rodriguez *et al.*, 1996).

Although the exact genetic basis for the inability of BALB/c mice to heal *L. major* infection is not clear, major histocompatibility complex (H-2) genes are not the main determinants for susceptibility to *L. major*; but it is closely linked to the susceptibility locus *Scl1* on mouse chromosome 11 (Blackwell *et al.*, 1994; Blackwell, 1996). This locus includes candidate genes *Nos2* encoding iNOS, and several monokines including MCP-1, MCP-2, MCP-3, MIP-1α, MIP-1β and RANTES, which are known to be produced by activated macrophages. In total, five regions in the mouse and human genome have been identified to carry genes associated with the ability to heal infections with a variety of species of *Leishmania* (Blackwell, 1996): *Scl-2* on chromosome 4 in the mouse may encode JAK-1 tyrosine kinase, which is thought to be responsible for the non-lesion phenotype observed in DBA/2 mice infected with *L. mexicana* (but not *L. major*); *Scl-1* on chromosome 11, as discussed earlier with regard to *L. major*, and the Th2 cluster on chromosome 11 encoding IL-4, IL-5 and IL-9, which is implicated in controlling later phases of *L. major* infection. Other genes linked to the ability of mice to heal *L. major* infection are *H-2* (human HLA) on mouse chromosome 17 encoding class II, class III and tumour necrosis factor α/β (TNFα/β). Finally, the positionally cloned mouse macrophage resistance gene *Nramp1* on chromosome 1 is thought to encode a membrane protein involved in the early stages of macrophage activation (Blackwell, 1996).

Immunity to Reinfection

Protection by vaccination depends on the induction of immunological memory. In contrast to antibodies, maternal memory T cells are not transmitted to the offspring. It is common knowledge that secondary immune responses to T-dependent antigens are more intense than primary responses (Cerottini and MacDonald, 1989). Although our understanding of immunological memory is improving (Gray, 1993; Sprent, 1994), there is still relatively little known about the molecular and cellular basis of this phenomenon. It is debated whether T-cell memory is due to the repeated restimulation of the immune system by persisting antigen or to the presence of long-lived memory T cells (Gray, 1993; MacKay, 1993; Müllbacher, 1994; Kündig *et al.*, 1996). The phenomenon of memory may even result from the combined effects of both long- and short-lived cells, as suggested by the apparent biphasic decay of memory (Celada, 1971). The nature of memory has been thought to reside either in an increased precursor frequency (Cerottini and MacDonald, 1989), or in a qualitatively altered differentiation state of memory T cells as compared with naive cells (MacKay *et al.*, 1990; Cerottini and MacDonald, 1989).

The characterization of the T cells involved in the recall response is an important prerequisite to identify the components of the immune system that must be sensitized by vaccination. Memory cells that reside in an animal after termination of the primary infection are different from naive cells; however, the mechanism by which they operate is still unresolved. In infections with *Mycobacterium tuberculosis*, the memory T cells present

after infection differ markedly from T cells activated during a primary infection. The re-encounter with the pathogen triggered a very restricted part of the T-cell repertoire during reinfection (Anderson and Heron, 1993).

Since there are no reliable markers expressed specifically on memory T cells, functional assays that determine directly the effects of specific memory cells on the fate of the pathogen represent a meaningful measure of T-cell activity (Kündig *et al.*, 1996). In the leishmaniasis model, the estimation of the parasite load present in an immune host after reinfection by limiting dilution analysis can be used to evaluate the nature of protective T-cell memory *in vivo* .

Experimental Models *In Vivo*

The particular inbred mouse strain and the actual number and type of activated T cells are, without any doubt, decisive determinants in the outcome of experimental infection with *L. major*. Transfer of high numbers of T cells derived from normal BALB/c mice into BALB/c nude mice resulted in non-healing disease after infection; when the nude mice were reconstituted with low numbers of T cells, they were able to resolve the lesions (Mitchell *et al.*, 1981b). Low numbers of T cells from chronically infected BALB/c mice were not protective in nude mice, and even abrogated the above described protective effect of low numbers of normal syngeneic cells (Mitchell *et al.*, 1981b; Mitchell, 1983). Cell transfer experiments into SCID mice confirmed the earlier observation that transfer of low numbers of T cells before infection results in healing, and transfer of high numbers in non-healing (Varkila *et al.*, 1993). The fact that reconstituted T-cell-deficient mice can develop both progressive disease or protective immunity after *L. major* infection indicated, almost 15 years ago, that qualitatively different T-cell responses can be induced in the *L. major* model. Translated into a more up-to-date terminology, these experiments showed that Th1 (healing) as well as Th2 (non-healing) cells can be induced.

The maturation of precursor Th cells into differentiated, functionally distinct Th1 or Th2 effector cells depends on numerous factors (Seder and Paul, 1994; Abbas *et al.*, 1996; Brown *et al.*, 1996), not all of which are fully understood and some of which are still controversial. Mice lacking a certain functional gene due to targeted gene disruption in embryonic stem (ES) cells are useful for the analysis of mechanisms resulting in the activation of naive $CD4^+$ precursor T cells to differentiate into mature functionally distinct Th1 or Th2 effector cells. Cytokine-deficient mouse mutants, generated by disruption of cytokine genes or cytokine receptor genes, represent clean models to analyse the contribution of cytokines to T-cell differentiation and to study the functions of cytokines *in vivo*. These models elegantly confirmed existing knowledge derived from antibody neutralization studies, or revealed surprising and unexpected new insights. The key role of IFN-γ has recently been confirmed; mice from healer strains lacking functional genes encoding either IFN-γ or the ligand binding chain of the IFN-γ receptor were unable to restrict the growth of *L. major* (Wang *et al.*, 1994; Swihart *et al.*, 1995). IFN-γ plays a decisive role

in mediating protective immunity *in vivo* by activating antimicrobial effector pathways in parasitized macrophages; it is important in the induction of the iNOS. The generation of NO via the NO pathway is required for parasite killing (Mauel *et al.*, 1991a,b; Oswald *et al.*, 1994). This was directly confirmed by the inability of iNOS knockout mice to restrict the growth of *L. major* parasites (Wei *et al.*, 1995).

IL-4 is considered to be a crucial cytokine for the development of Th2 responses and the non-healing cutaneous leishmaniasis in BALB/c mice (Liew and O'Donnel, 1993; Reiner and Locksley, 1995). The constitutive expression of IL-4 in transgenic mice from a healer strain interfered with the resolution of cutaneous lesions (Leal *et al.*, 1993), indicating a role for IL-4 in the progressive disease. Neutralization of IL-4 in the early phase of *L. major* infection allows non-healer BALB/c mice to resolve their cutaneous lesions and to express a Th1 cytokine profile (Sadick *et al.*, 1990; Müller *et al.*, 1991; Chatelain *et al.*, 1992; Nabors and Farrell, 1994a). This contrasts with the surprising findings obtained with *L. major*-infected genetically pure IL-4 BALB/c knockout mice, generated from a BALB/c-derived ES cell line (Noben-Trauth *et al.*, 1996), which did not resolve their lesions or switch to a Th1 cytokine profile. However, other investigators arrived at different conclusions. In their hands, IL-4-deficient mice (F2:129Sv × C57BL/6), back-crossed for six generations onto the BALB/c background, as well as the genetically pure BALB/c IL-4 knockout mice were found able to control *L. major* infection (Kopf *et al.*, 1996). The reason for this discrepancy is not fully understood at present. The different isolates of *L. major* used for these studies could be responsible for the different results.

Redirection of Non-healing Responses

The *L. major* model offers remarkable possibilities to alter immune responses and interfere drastically with the normal development of the disease. The non-healing disease of BALB/c mice serves as a useful model to study ways and mechanisms resulting in a correction of undesired immune responses and deviating the response in a desired direction.

The non-healing response of BALB/c mice to *L. major* infection can be manipulated at the time of infection in such a way that these mice are able to fight infection, to resolve their lesions and reduce the parasite load. Manipulations like sublethal irradiation (Howard *et al.*, 1981), intravenous immunization (Farrell *et al.*, 1989), low-dose infection (Bretscher *et al.*, 1992), treatment with anti-IL-2 monoclonal antibody (mAb) (Heinzel *et al.*, 1993a), recombinant IL-12 (Sypek *et al.*, 1993; Heinzel *et al.*, 1993b; Afonso *et al.*, 1994), anti-CD4 mAb (Titus *et al.*, 1985a; Sadick *et al.*, 1987; Müller *et al.*, 1988, 1989, 1991; Liew *et al.*, 1989), and anti-IL-4 mAb injection (Sadick *et al.*, 1990) can reverse the non-healer phenotype of BALB/c mice. It is interesting that injection of recombinant IFN-γ does not reverse the progressive disease and does not restore Th1 development (Sadick *et al.*, 1990; Scott, 1991). IL-12 is the only cytokine known that results in a stable reversal of non-healing *L. major* infections in BALB/c mice (Sypek *et al.*, 1993; Heinzel *et al.*, 1993b; Afonso *et al.*, 1994). The immunological

manipulations such as treatment with IL-12 or with antibodies to neutralize IL-4 or the transient elimination of $CD4^+$ helper cells must be done in the early phase of infection to be effective. The ability to redirect the immune response of the extremely sensitive non-healer BALB/c mice clearly shows that the T cells of these mice do have the ability to mount a protective immune response. These treatments can shift the balance of Th subsets and consequently direct immune responses to the Th1 differentiation pathway. For example, a single injection of anti-CD4 mAb enables BALB/c mice to control parasite proliferation, heal their *L. major* infection, develop immunity, and resist a subsequent challenge infection (Müller *et al.*, 1991). The precise mechanism of action of the anti-CD4 mAb treatment is not completely understood; however, since most immune interventions are only effective when given either before infection or in the early phase of infection, i.e. in the first 2 weeks, they are thought to influence the differentiation of naive Th precursors into polarized Th1 or Th2 effector cells. Furthermore, the capacity of anti-CD4 mAb-treated mice to heal infection has been correlated with the abrogation of the IL-4 burst (Reiner *et al.*, 1994; Reiner and Locksley, 1995). Even established polarized Th2 responses can be switched in *L. major* infected BALB/c mice by combining conventional drug treatment that results in the reduction of the parasite load with immunomodulatory agents that direct responses towards the Th1 pole (Nabors *et al.*, 1995; Nabors, 1997).

Due to its public health importance and its popularity as a basic immunological model system to analyse Th cell subset differentiation, and cross-regulation of immune responses by cytokines, leishmaniasis has been studied extensively. Most immunological techniques described in other chapters in this book are used to study experimental leishmaniasis, so we shall focus our contribution on methods that are specific for the parasite model. However, even there, the range of applications of the experimental leishmaniasis model is so wide that we cannot cover all methods used in this field, so we will present those used in our laboratory. *Leishmania* parasites are not simple, inert and invariable antigens, they are highly complex eukaryotic organisms. The induction of the immune response by the parasite is of crucial importance for the developing response; therefore, we consider it important to describe methods aiming at a better standardization of the infection. These include the maintenance of the parasites, the initiation of infections and methods to evaluate the development of disease, and the parasite load. A statistical programme is described for the evaluation of the parasite burden in infected tissues.

◆◆◆◆◆ METHODS

Parasite Maintenance and Isolation

In the following paragraphs we describe in detail the materials as well as the preparative steps necessary for the handling of *Leishmania*.

Maintenance media

L. major promastigotes can be maintained in different media. The most commonly used are Schneider's *Drosophila* medium (Gibco), Grace's insect cell culture medium (Gibco), and a biphasic system consisting of a liquid phase of Dulbecco's modified Eagle's medium (DMEM, Cellgro, Mediatech) over a solid layer of rabbit blood agar. In our laboratory we use the biphasic culture. The DMEM used routinely to maintain the parasites in culture is supplemented with:

- 10% complement inactivated fetal bovine serum (FBS)
- 50 IU ml^{-1} penicillin
- 50 µg ml^{-1} streptomycin
- 292 µg ml^{-1} L-glutamine
- 4.5 mg ml^{-1} glucose.

The same medium is used for the parasite-limiting dilution assay (LDA medium).

The FBS is necessary for optimal culture conditions. However, some batches of FBS can inhibit the parasite growth, so careful screening is necessary.

Blood agar

To prepare 100 ml blood agar medium, the following material is necessary:

- 3.0 g nutrient agar (Difco, Detroit, MI)
- 0.6 g NaCl
- 100 ml bidistilled water
- 10 ml rabbit blood (9 ml blood + 1 ml 4% sodium citrate; Merck)
- 5 ml 30% glucose in phosphate buffered saline (PBS)
- 70 ml polystyrene tissue culture flask.

1. Autoclave the mixture of agar, water and NaCl for 20 min.
2. Cool the agar to about 45°C.
3. Place into a 45°C water bath.
4. Add 10 ml rabbit blood and 5 ml 30% glucose.
5. Distribute 5 ml agar mixture on the flat side of the 70-ml polystyrene tissue culture flask.
6. Allow to solidify and cool at room temperature.
7. Tightly close the flasks.
8. Keep at 4°C for up to 4 weeks.

Parasite counting

Promastigotes move actively and must be fixed for counting. Before fixing, parasites are washed as follows:

1. Centrifuge for 5 min at 60*g* to pellet debris.
2. Transfer the supernatant in a new tube.
3. Wash the supernatant twice at 1400*g* for 10 min.
4. Resuspend the parasites in the maintenance medium and fix them using one of the following two solutions:
   - 2% formaldehyde in PBS
   - Hayem's solution (0.5 g $HgCl_2$, 1.0 g NaCl, 5.0 g Na_2SO_4, 200 ml H_2O).
5. After resuspending the parasites in DMEM, we usually dilute them 1/100 in 2% formaldehyde in PBS and count them in a haemocytometer.

Parasite maintenance: *in vivo* and *in vitro*

L. major is a hazard group 2 pathogen and should be treated accordingly. It is easy to handle *in vitro* and *in vivo*; however, it is important to pay attention to a few points relating to the virulence and infectivity of these organisms. The maintenance of *L. major* in culture has an innate problem in that the parasite undergoes an evolution different from that in the natural environment where it is cycled through the sandfly prior to infection in a new vertebrate host. A decrease in virulence is the dominant characteristic of a long-term maintenance *in vitro*. To maintain their virulence, a monthly passage *in vivo* is necessary. Many strains of mice can be used for this purpose. In our laboratory, we use BALB/c mice and infect them in the hind foot pad with *L. major* LV39 (MRHO/SU/59/P strain) in a final volume of 50 µl. The mice are killed 2–4 weeks after infection and the parasites are isolated from the foot-pad lesion.

Parasite isolation

L. major is isolated from the infected foot pad as follows:

1. Swab the skin of the dead mouse with 70% ethanol.
2. Make an incision of the skin around the ankle.
3. Cut the toes of the foot pad.
4. Carefully remove the skin and necrotic tissue of the infected foot pad.
5. Cut the foot pad just above the joint.
6. Place the foot pad in a sterile Petri dish.
7. Cut the foot pad in several small pieces.
8. Transfer the pieces into a sterile Tenbroeck glass homogenizer.
9. Gently homogenize the tissue in 5 ml medium.
10. Transfer the suspension in a blood agar flask.
11. Incubate at 26°C, in a humid atmosphere, 5% CO_2 in air, to allow transformation of amastigotes into promastigotes (the parasites can also transform and grow in tightly closed flasks in a dry incubator).
12. After 3–5 days, the suspension is washed as before.
13. Resuspend the parasites in 5 ml maintenance medium and transfer the suspension in a new flask.

Parasite maintenance

Once isolated, the parasites should be maintained for a maximum of 4 weeks with the minimal number of dilutions *in vitro* possible. Like other unicellular organisms, *L. major* have lag, log and stationary phases, and promastigote populations during these phases are not uniform with respect to infectivity. During the log phase, parasites are motile and dividing cells. When injected in a foot pad of a non-healer mouse, lesions will start to appear only 8–10 weeks after the infection. However, infection with stationary phase *L. major* will result in visible lesions as soon as after 1 week. These parasites are stationary and non-dividing. The cultures are started with 10^6 parasites ml^{-1}. After a few days, the parasites will reach the stationary phase. Leishmanial growth can be monitored by daily counting of the cells and by microscopic observation of the cultures. Stationary parasites are washed, counted, and a new culture is started with 10^6 parasites ml^{-1} in a new blood agar flask.

Cryopreservation of parasites

L. major promastigotes can be cryopreserved by using the same techniques as for other eukaryotic cells. For successful cryopreservation, it is important to use parasites from the first passage *in vitro* after a new isolation and to freeze them in a late log phase. We cryopreserve promastigotes in the following way:

1. Wash the parasites as described above.
2. Adjust them to 1×10^8 cells ml^{-1} in FBS containing 10% of dimethyl sulfoxide in a final volume of 1 ml per tube. The tubes are placed in a styrofoam box and immediately placed at -70°C for 24 h, and are then stored in the vapour phase of liquid nitrogen until further use.

As for other eukaryotic cells, the parasites have to be thawed quickly. We thaw the vial in a 37°C water bath and distribute the cells dropwise in a tube containing medium at room temperature. The cells are washed once at 1400*g* for 10 min, resuspended in their culture medium, and placed in a blood agar flask.

It is imperative to passage the thawed batch of parasites in a mouse. In our experience, a clear reduction in virulence was noted with extended time in storage.

Isolation of metacyclic promastigotes for infection: peanut agglutinin agglutination

Metacyclic *L. major* parasites represent the infectious form of the parasite. Log phase procyclic and stationary phase metacyclic promastigotes of *L. major* differ in the composition of the repeating phosphorylated saccharide

unit of LPG. The repeat units of LPG from log phase *L. major* contain terminal β-galactose residues and these galactose residues account for the agglutination by the lectin peanut agglutinin (PNA). The repeat units of metacyclic promastigotes terminate predominantly with α-arabinose, which is not a ligand for the lectin (Sacks *et al.*, 1985; Sacks and Da Silva, 1987; Turco and Descoteaux, 1992). Metacyclic promastigotes lose their ability to bind PNA, and a technique using this differential binding has been developed to purify infective stages of *L. major* from cultures (Sacks *et al.*, 1985).

To isolate metacyclic *L. major* parasites, the following material is necessary:

- PBS
- PNA isolated from *Arachis hypogae* (Vector Laboratories; Sigma).

Isolation

1. Wash the stationary phase parasites three times with PBS.
2. Adjust the parasites to 1×10^8 cells ml^{-1} in PBS.
3. Add an equal volume of PNA (100 μg ml^{-1}) in PBS.
4. Incubate for 1 h at room temperature.
5. To isolate the non-agglutinated parasites, two methods can be used:
   - Harvest the suspension and carefully layer it on top of the same volume of PBS containing 50% of FBS. Incubate for 30 min at room temperature. Carefully harvest the parasites remaining above the interphase. Wash three times in PBS and count.
   - Harvest the suspension and centrifuge at 80*g* for 5 min. Carefully harvest the supernatant, wash three times in PBS and count.

Factors Influencing the Outcome of Infection

Route of infection

We usually inoculate the desired number of infective *L. major* promastigotes subcutaneously in one hind foot pad in a final volume of 50 μl PBS. The route of entry of the *L. major* promastigotes is one of the factors that influence the nature of the developing immune response. Nabors and Farrell (1994b) showed that SWR mice display a non-healing response to *L. major* comparable to that of BALB/c mice when the parasites are inoculated subcutaneously at the base of the tail. In contrast, if infected subcutaneously in the foot pad with the same number of parasites, the SWR mice were able to control their lesion.

Mouse strain	Route of parasite entry	Form of disease
SWR	s.c., base of tail	Non-healing
SWR	s.c., foot pad	Healing

s.c., subcutaneous

Although the two routes of injection lead to different outcomes of infection in these genetically identical mice, there was no apparent difference in the early cytokine levels (Nabors and Farrell, 1994b). In the SWR model, infection at either site leads to an early mixed Th1/Th2 cytokine response, but only the foot-pad infection leads to healing of the lesion, indicating that factors other than the early cytokine response influence the development of disease.

The development of progressive disease is not restricted to BALB/c mice and is not exclusively determined by the genetic composition of the infected host. C57BL/6 mice, one of the prototype healer strains, can resolve cutaneous lesions developing after subcutaneous infection. However, when infected intravenously with *L. major*, mice from this strain developed non-healing disease and were unable to mount a DTH response (Scott and Farrell, 1982). Local differences in the skin temperature might influence the growth of *L. major* amastigotes and the responsiveness of macrophages to cytokines, and contribute to the expression of site-specific immunity.

Infectious dose

It is important to bear in mind that mice that display a healer or non-healer phenotype when infected with a given dose, and species of *Leishmania* may behave quite unexpectedly when infected with a different number or species (or even strain) of parasite. Thus, subcutaneous injection of 10^6 *L. major* promastigotes in one foot pad of BALB/c mice results in expanding cutaneous lesions at the site of inoculation, visceralization of infection and death of the host (Mitchell *et al.*, 1981b), although the time at which death occurs depends on the parasite strain and dose (Mitchell *et al.*, 1981a). However, when infected with a low dose of virulent *L. major* promastigotes, the BALB/c mice display all the characteristics of a healer strain, in that they fail to develop a lesion at the site of infection or only a small one that resolves spontaneously, and become solidly immune to reinfection (Bretscher *et al.*, 1992; Menon and Bretscher, 1996). This suggests that the number of infectious parasites affects the development of T-cell subsets (Doherty and Coffman, 1996).

Enumeration of Viable Parasites by Limiting Dilution Assay

The most commonly used index for disease progression in *L. major*-infected mice as well as for the determination of the effects of immunotherapies is the measurement of the size of cutaneous lesions that develop at the site of parasite inoculation. However, lesion size does not always correlate with the number of viable parasites within the lesion. A technique has been developed by Titus and co-workers (Titus *et al.*, 1985b; Lima *et al.*, 1997) to determine the degree of parasitism in different infected tissues. An example of an infected foot pad is described here, but the same technique can be used for different organs.

Preparation of blood agar plates

For the determination of the number of viable parasites, the tissue homogenates from infected mice need to be plated on blood agar plates.

1. Prepare the blood agar as described above.
2. Distribute 50 µl agar mixture per well in a slant (Fig. 1) with a repeater pipetter (Eppendorf) in 96-well flat-bottom tissue culture plates.
3. Allow to solidify and cool at room temperature.
4. Keep in a humid box at 4°C for up to 4 weeks.

Preparation and plating of tissue homogenates

To enumerate the number of viable parasites present in the lesion, the following technique is used:

1. Determine the weight of a Petri dish.
2. Prepare the foot pad as described for the parasite isolation.
3. Weigh the Petri dish with the foot pad.
4. Calculate the weight of the foot pad.
5. Cut the foot pad and homogenize it as described earlier.
6. Prepare 8–12 serial 10-fold dilutions of the foot-pad homogenate in a final volume of 2.5 ml (see Fig. 1).
7. Distribute 100 µl per well of each dilution in at least 16 replicate wells, changing pipettes between each dilution (see Fig. 1).
8. Incubate the plate at 26°C, in a humid atmosphere, 5% CO_2 in air, to allow transformation of amastigotes into promastigotes (the parasites can also transform and grow in plates sealed with Parafilm in a dry incubator).
9. After 10 days, the assay is read by scoring the number of positive wells (presence of motile parasites) and negative wells (absence of motile parasites). An example is illustrated in Table 1.

Note: If the parasite load is determined in lymphoid organs, count the cells and use the number of cells instead of the tissue weight for the frequency estimation.

Table 1. Results of the microscopic scoring of positive and negative wells

	1	2	3	4	5	6	7	8		1	2	3	4	5	6	7	8
A	+	+	+	+	+	+	−	−	A	+	+	+	+	+	+	−	−
B	+	+	+	+	+	+	−	−	B	+	+	+	+	+	+	−	−
C	+	+	+	+	+	−	−	−	C	+	+	+	+	+	+	−	−
D	+	+	+	+	+	+	−	−	D	+	+	+	+	+	+	−	−
E	+	+	+	+	+	+	−	−	E	+	+	+	+	+	+	+	−
F	+	+	+	+	+	+	−	−	F	+	+	+	+	+	+	−	−
G	+	+	+	+	+	+	+	−	G	+	+	+	+	+	+	+	−
H	+	+	+	+	+	+	−	−	H	+	+	+	+	+	+	−	−

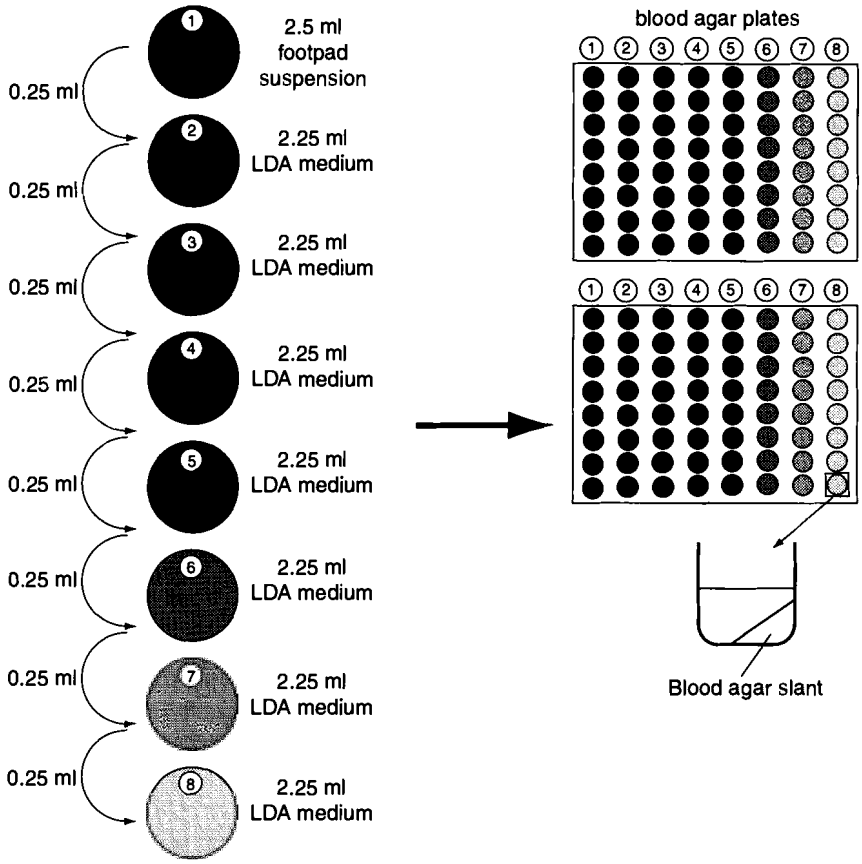

Figure 1. Plating of tissue homogenate.
1. Carefully mix well 1 with a 1-ml pipette.
2. Remove 0.25 ml and transfer it to well 2.
3. Using the same 1-ml pipette, remove 0.8 ml from well 1.
4. Distribute 0.1 ml into eight replicates of one blood agar plate.
5. Repeat steps (3) and (4), but distribute into the second plate.
6. Change the pipette, and repeat the procedure for wells 2 and 3.

Plating efficiency

To determine the plating efficiency of the limiting dilution assay (LDA), a control plate consisting of serial dilutions of a known number of parasites has to be set up. The model of dilutions depicted in Fig. 1 can be used. We perform six serial 10-fold dilutions, starting with a concentration of 1×10^4 *L. major* per well. After 10 days, the assay is read by scoring the number of positive and negative wells .

Analysis of the Parasite LDA Results

The objective of the LDA is to determine the number of viable parasites in a given tissue. As direct counting is not possible, staining techniques need

to be applied to detect intracellular *Leishmania* parasites. However, these techniques do not allow us to distinguish between live and dead parasites. Therefore, plating infected tissues under conditions optimal for parasite transformation and growth and determining the number of transformed, viable, motile promastigotes in these cultures permits us to reach this goal. The raw data obtained will be analysed using a SAS program to evaluate the number of parasites in the tissue. Statistical analysis of experimental data is used to estimate the frequency of parasites in the tissue. Based upon the estimated frequency, simple arithmetic produced an estimate of the total number of parasites in the initial amount of tissue.

Consistent with the methods detailed by Taswell (1987), a SAS PROC IML program was written to calculate frequencies, test statistics and descriptive statistics. The single-hit Poisson model (SHPM) described was assumed to represent the distribution of organisms in the dose and the ability to detect parasites. The minimum χ^2 (MC) iterative method was chosen to estimate the frequency of parasites, χ^2 tests were used to validate the assay, and the final estimate and confidence levels were reported for completeness. The SAS program was validated using data published in Taswell (1987). Notation is also consistent with that used by Taswell (1987). Calculations were performed on an IBM 9121 running VM/ESA 1.1; CMS Level 8, Service Level 105. SAS was version 6.09, TS450. The program is given at the end of this chapter.

Validity models

d	number of dose levels
n_d	number of wells in the dth dose level
p_d	observed negative response frequency ($= r_d/n_d$)
r_d	number of negatively responding wells in the dth dose level
w_d	regression weights
Y_d, X_d	dependent and independent variables
y_d, x_d	deviations from sample means of Y_d and X_d
α_1, α_2	constant terms
β_1, β_2	slope parameters
λ_d	known dose of the dth dose level in nanograms
ϕ	unknown relative frequency to be estimated

The assay validity test (AVT) was conducted by testing the estimate of the slope parameter β_m

$$\hat{\beta}_m = \frac{\Sigma w_d x_d y_d}{\Sigma w_d x_d^2} \tag{1}$$

First, based on the least squares regression model

$$Y_d = \alpha + \beta_d X_d$$

two weighted, generalized linear forms are specified

$$\ln \hat{\phi}_d = \alpha_1 + \beta_1 \lambda_d \tag{2}$$

$$\hat{\phi}_d = \alpha_2 + \frac{\beta_2}{\lambda_d} \tag{3}$$

For (2),

$$w_d = \frac{n_d p_d (\ln p_d)^2}{1 - p_d} \tag{4}$$

and for (3)

$$w_d = \frac{n_d p_d \lambda_d^2}{1 - p_d} \tag{5}$$

Next, the weighted means of Y_d and X_d are calculated

$$\bar{Y}_w = \frac{\Sigma w_d Y_d}{\Sigma w_d} \text{ and } \bar{X}_w = \frac{\Sigma w_d Y_d}{\Sigma w_d}$$

and then used to calculate deviations from the sample mean

$$y_d = Y_d - \bar{Y}_w \text{ and } x_d = X_d - \bar{X}_w$$

Finally, calculate the χ^2 statistic

$$\chi^2 = \frac{(\Sigma w_d x_d y_d)^2}{\Sigma w_d x_d^2} = \hat{\beta}_m \Sigma w_d x_d y_d \tag{6}$$

with one degree of freedom to test the hypothesis that $\hat{\beta}_m = 0$

The estimate validity test (EVT) tested the validity of ϕ, the final estimate of ϕ, by substituting

$$\hat{p}_d = \exp(-\hat{\phi}\lambda_d) \tag{7}$$

for p_d in (5) and (6) above. The larger of (4) from (2) or (3) with a correspondingly smaller p-value was then reported as the χ^2 test statistic with 1 degree of freedom for determining estimate validity.

Hypothesis testing

Two hypotheses were tested; in both cases failing to reject the null hypothesis that $\beta_m = 0$ was desired. The SHPM assumes that the presence of only a single organism is sufficient for detection, and independence between the sample frequencies and the dose level are consistent with that assumption. The AVT tests the fit between the model and the data. The EVT adds information on the estimated frequency to the AVT test.

Estimation

The MC estimate $\hat{\phi}$ of the unknown frequency ϕ was calculated as the value that minimizes

$$\chi^2 = \Sigma\left[\frac{(r_d - n_d e^{-\phi\lambda_d})^2}{n_d e^{-\phi\lambda_d} - (1 - e^{-\phi\lambda_d})}\right] \tag{8}$$

where $\hat{\phi}$ is determined iteratively from Newton's method

$$\hat{\phi}_{i+1} = \hat{\phi}_i - \frac{(\partial\chi^2/\partial\phi)}{(\partial^2\chi^2/\partial\phi^2)}\bigg|_{\hat{\phi}_i} \tag{9}$$

where $\hat{\phi}_i$ is the estimate from the ith iteration. The first partial derivative is

$$\left.\frac{\partial \chi^2}{\partial \phi}\right|_{\hat{\phi}_i} = \Sigma\left[\frac{n_d\lambda_d e^{-\phi\lambda_d}(2r_d - n_d) + r_d^2\lambda_d(e^{-\phi\lambda_d} - 2)}{n_d(1 - e^{-\phi\lambda_d})^2}\right]\Bigg|_{\hat{\phi}_i} \quad (10)$$

and the second partial derivative is

$$\left.\frac{\partial^2 \chi^2}{\partial \phi^2}\right|_{\hat{\phi}_i} = \Sigma\left[\frac{n_d\lambda_d^2(n_d - 2r_d)(e^{-\phi\lambda_d} - e^{-3\phi\lambda_d}) + r_d^2\lambda_d^2(e^{-\phi\lambda_d} - 4 + 7e^{-\phi\lambda_d} - 4e^{-2\phi\lambda_d})}{n_d(1 - e^{-\phi\lambda_d})^4}\right]\Bigg|_{\hat{\phi}_i} \quad (11)$$

The estimated variance is calculated as twice the reciprocal of the second partial derivative evaluated at the final estimate $\hat{\phi}$

$$\hat{V}(\hat{\phi}|\phi) = \left.\frac{2}{(\partial^2\chi^2/\partial\phi^2)}\right|_{\hat{\phi}} \quad (12)$$

Example

Two foot pads (weight 0.165 g) were homogenized, resuspended in 10 ml LDA medium (1 650 000 ng tissue in the first 100 µl) and plated as described above. After 10 days, the assay was read by scoring the number of positive and negative wells. The results are illustrated in Tables 1 and 2.

Table 2. Example of responding groups

Dilution	Negative wells	ng tissue/100 µl
1. 1/1	0	1 650 000
2. 1/10	0	165 000
3. 1/100	0	16 500
4. 1/1000	0	1650
5. 1/10 000	0	165
6. 1/100 000	1	16.5*
7. 1/1 000 000	13	1.65*
8. 1/10 000 000	16	0.165

*Responding group

Table 3 presents the data from a single experiment. In Table 4, the p values for the AVT are both large, meaning that the null hypothesis is not rejected. Table 5 lists the statistics from the EVT, which also fail to reject the null hypothesis. Final estimates of the unknown frequency, variance, confidence interval, and total parasites are in Table 6.

Table 3. Experimental data: tissue, 165 000 000 ng; $n_d = 16$

d	r_d	λ_d
1	1	16.5
2	13	1.65

Table 4. Assay validity test

	Model (2)	Model (3)
χ^2 AVT	0.183 152 9	0.203 666 7
p values of AVT	0.668 677 6	0.651 777 3

Table 5. Estimate validity test

χ^2 EVT	0.212 245 4
p value of EVT	0.645 013 2

Table 6. Estimates and descriptive statistics

Estimated frequency	0.153 324 5
Estimated variance	0.002 319 5
95% upper confidence level	0.247 721 2
95% lower confidence level	0.058 927 9
1/Frequency	6.522 114 5
Estimated number of parasites per lesion	12 649 272

◆◆◆◆◆◆ STUDIES OF THE HOST RESPONSE TO INFECTION

Measuring the Course of Experimental Infection

Many studies of experimental cutaneous leishmaniasis rely on the measurement of the changes of the local swelling which develops at the site of parasite inoculation to follow the course of infection. The replicating parasites, inflammatory responses of the infected host and pathological changes due to infiltrating cells contribute to the expression of the lesion. The lesion size can be due to increased parasite growth or to inflammatory or pathological reactions (Hill *et al.*, 1983; Titus *et al.*, 1985b). Therefore, monitoring only the increase or decrease in the cutaneous lesion may give inaccurate or even false impressions of the true progress of the disease (Hill *et al.*, 1983; Vieira *et al.*, 1996). Similar differences in lesion sizes can be accompanied by great differences in the number of viable parasites. Lesions have been described in which there were no parasites, and parasites could be found when there were no detectable lesions (Hill *et al.*, 1983; Titus *et al.*, 1985b; Vieira *et al.*, 1996). The combined use of monitoring the size of the cutaneous lesions and the determination of the parasite burden in the infected tissue reflect more accurately the status of disease, the effect of vaccines and immunotherapy.

Evaluation of cutaneous lesions

After subcutaneous inoculation of infectious *L. major* parasites in one hind foot pad, the development of the infection can be followed by determining the degree of local swelling at the site of inoculation. Infection into the foot pads are widely used. The advantage of this route is that in immunocompetent individuals, the infection remains mainly restricted to the site of inoculation and the uninfected, contralateral foot pad can be used as a control. To determine the size of the cutaneous lesions, the actual thickness of the infected and the non-infected contralateral foot pad are measured

at regular intervals (for example, on a weekly basis). The size of the foot pads can be determined with a suitable dial caliper (Kröplin, Schlüchtern, Germany). The lesion size is calculated by substracting the thickness of the non-infected contralateral foot pad from the thickness of the infected foot pad. An example of the lesion size during the course of *L. major* infection in healer and non-healer mice is given in Table 7 and Fig. 2.

Table 7. Evolution of lesions in healer and non-healer mice

Weeks after infection	BALB/c (foot pad size ± SEM)	CBA (foot pad size ± SEM)
1	0.25 ± 0.08	0.30 ± 0.04
2	0.48 ± 0.07	0.91 ± 0.04
3	1.02 ± 0.06	1.10 ± 0.06
4	1.80 ± 0.30	1.09 ± 0.09
5	2.43 ± 0.13	0.68 ± 0.06
6	3.80 ± 0.10	0.50 ± 0.02
7	4.65 ± 0.75	0.32 ± 0.04

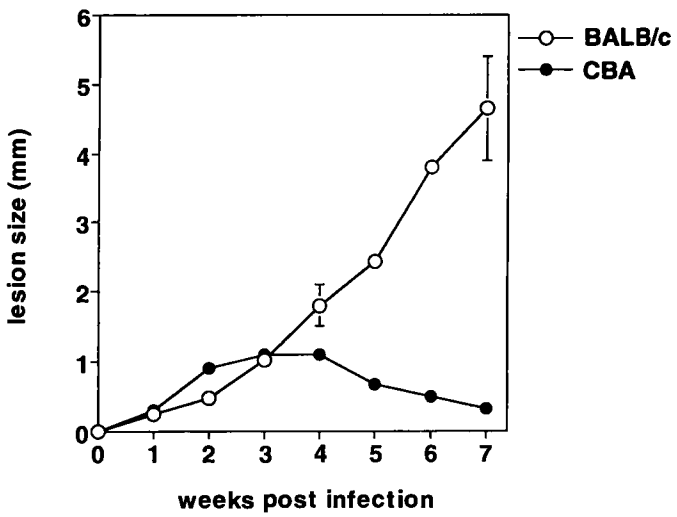

Figure 2. Lesion-size development during *L. major* infection.

As already mentioned, the course of experimental infection should not only be evaluated by the lesion development, but also by enumeration of the number of viable parasites present in the lesions at several time points during infection.

T-Cell and Cytokine Responses

$CD4^+$ Th cells have been divided into the Th1 and Th2 subset based on the production of a distinct panel of cytokines: Th1 cells secrete IFN-γ, IL-2 and

TNFβ; Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13. The elucidation of the steps leading to the priming of these cells is a major focus of immunology, and it is widely accepted that cytokines play a key role in this priming. Cytokines can cross-regulate each other's production and this influences the function of Th1 and Th2 cells. Experimental cutaneous leishmaniasis induced by infection with *L. major* is one of the best studied models in which selective activation of Th1 or Th2 cells occurs (Liew and O'Donnel, 1993; Reiner and Locksley, 1995). The induced pattern of cytokines is used as an indicator for the type of activated Th-cell subset. The cytokine pattern can be determined by different methods. The induction of mRNA by quantitative reverse transcriptase polymerase chain reaction (RT-PCR), the intracellular staining with fluorescent anticytokine antibodies, the estimation of the frequency of cytokine secreting cells by enzyme-linked immunosorbent assay spot (ELISPOT) and the determination of the amount of cytokine secretion by enzyme-linked immunoassay (ELISA) or bioassays are possible methods. All the different methods used for cytokine analysis as well as for T-cell isolation, phenotyping, stimulation and proliferation are covered in detail in chapters II.2.1, II.2.2, II.2.4 and II.3.2.

However, we would like to describe briefly the stimulation conditions used in our laboratory for the detection of cytokines by ELISA, bioassays and ELISPOT. After infection of mice with *L. major*, T cells will be activated and migrate. Therefore, the pattern of cytokines secreted by T cells is determined in at least two distinct lymphoid organs: the spleen and the lymph nodes draining the site of infection. Suspensions of total spleen and lymph nodes cells are adjusted to 5×10^6 to 1×10^7 cells ml^{-1} and stimulated in 24-well culture plates with the homologous antigen or immobilized anti-CD3 mAb (Leo *et al.*, 1987). Cells without additional stimulation *in vitro* are always used as a control. To restimulate specific T cells *in vitro*, live *L. major* promastigotes are rendered replication-incompetent by 2-min irradiation with ultraviolet light and are used at 4×10^6 parasites per millilitre of lymphocytes. To measure the maximum T-cell response, we use plate-bound anti-CD3 mAb. The antibody (5 μg) is added to the plates in a final volume of 200 μl PBS and allowed to bind to the plastic for 2 h at 37°C. Then, the wells are washed three times with PBS and the cells are added. IFN-γ is easily detected after 24 h, but Th2 cytokines require a longer incubation time. Therefore, we harvest the supernatants after 48–72 h of stimulation, aliquot them, and store them at –70°C until cytokine determination.

Antibody Responses and Immunoglobulin Isotypes

The antibody response in the cutaneous form of human leishmaniasis appears to reflect both the parasite load and the chronicity of infection (Ulrich *et al.*, 1996). Analysis of the composition of Ig subclasses in the serum of infected mice constitutes a useful read-out system for the activation of Th1 or Th2 cells *in vivo* and can be used as a tool to evaluate the effect of immunomanipulation or vaccination *in vivo* in an intact animal (Ulrich *et al.*, 1996).

For the determination of Ig isotypes, blood should be collected at different times after infection. The blood can be drawn from the orbital sinus of the mouse, the axillary plexus, from the tail vein or by cardiac puncture. We collect blood routinely from the orbital sinus of mice, allow the fresh blood to clot, centrifuge once, remove the clear serum and store it in small aliquots at –20°C until use.

The Ig isotypes present in the sera are determined by ELISA. We use commercially available kits or matched antibody pairs and standards (PharMingen, San Diego, CA, USA; Southern Biotechnology Associates, Birmingham, AL, USA) and follow the protocols provided by the producers. The optimal concentrations of capture and detecting antibodies must be determined for each batch, and the appropriate dilution of the sera to be tested will differ significantly, and thus also need to be determined in preliminary titration experiments.

Macrophage Activation, NO Production and Parasite Killing

Macrophages are the only mammalian cells that support the growth of *Leishmania* parasites. Therefore, healing of infection is clearly dependent upon the ability of macrophages to kill the intracellular parasites. Macrophages activated by exposure to cytokines acquire microbicidal activity due to the induction of the expression of iNOS and the generation of NO, the major effector molecule for the destruction of the intracellular *Leishmania* (Mauel *et al.*,1991a; Oswald *et al.*, 1994; Wei *et al.*, 1995).

Peritoneal, splenic or bone marrow derived macrophages can be infected with *L. major*, activated, and used to determine the leishmanicidal activity of macrophages. Detailed methods for the isolation and activation of macrophages of different origin are described in the chapter by Haworth and Gordon in Section II. We describe briefly the use of bone marrow derived macrophages.

Activation of bone marrow macrophages

Bone marrow (BM) culture derived macrophages are obtained by differentiation of BM precursor cells *in vitro*. BM precursor cells are flushed from the femurs and tibiae of normal mice and cultivated at a concentration of 5×10^5 cells ml^{-1} in hydrophobic Teflon bags (Heraeus, Hanau, Germany) (Freudenberg *et al.*, 1986). After 10 days of culture, BM-derived macrophages are harvested by centrifugation at 4°C, washed extensively with cold DMEM, seeded in 96-well microtitre plates at a density of 10^5 macrophages in 100 µl serum-free DMEM and cultured overnight to allow the cells to adhere. After adherence, BM-derived macrophages are infected at a ratio of 1 : 5 with *L. major* promastigotes. Plates are washed 6 h later to remove non-phagocytosed parasites, and infected macrophages are activated with 20 ng per well lipopolysaccharide, 10 U per well IFN-γ and 200 U per well TNFα. After 48 h, culture supernatants are removed and the levels of NO_2^- are tested by the Griess assay (see below). The remaining macrophages can be lysed and the survival of parasites can be determined by colorimetric quantitation (see below).

Nitrite determination

Nitrite (NO_2^-) and nitrate (NO_3^-) are stable oxidation products of NO, and NO_2^- accumulation is used as an indicator of NO production. The Griess reaction is a fast and simple colorimetric assay for nitrite. Nitrite in cell-culture supernatants is measured by the method of Ding *et al.* (1988) by adding to the culture supernatants an equal volume of Griess reagent (1% sulfanilamide and 0.1% n-(1-naphthyl)ethylenediamine dihydrochloride in 5% H_3PO_4; Sigma, St Louis, MO, USA). After 10 min at room temperature, the absorbance at 570 nm is measured with a spectrophotometer. NO_2^- concentration is determined using $NaNO_2$ dissolved in DMEM as a standard and DMEM alone as a blank.

Nitrate does not react in the Griess reaction. In the blood, NO_2^- is rapidly converted into NO_3^- by haemoglobin, so in blood or urine samples from infected mice NO_3^- must be reduced to NO_2^- with nitrate reductase for measurement by this assay.

Release and colorimetric quantitation of surviving intracellular *L. major*

BM-derived macrophages infected *in vitro* are activated as described above and the survival and killing of parasites is determined (Kiderlen and Kaye, 1990). After incubation for 48–72 h, the macrophages are washed once with 37°C *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES)-buffered RPMI (RPMI 1640, Gibco) containing 0.008% w/v sodium dodecyl sulfate (SDS) (lysis medium) and incubated with 100 µl fresh lysis medium for 7–20 min. During this time, macrophage disintegration has to be monitored regularly with an inverted microscope. Once host cell lysis is complete, 150 µl per well HEPES-buffered RPMI containing 17% fetal calf serum (FCS) is added to remove the SDS. The lysates are then incubated in a CO_2 incubator (5% CO_2 in air) for 48–72 h at 26°C to allow the parasites to transform to the promastigote stage.

The relative number of viable *Leishmania* per well is determined by a modified version of the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) (Mosmann, 1983). The test is based on the ability of viable cells to metabolize the yellow water-soluble tetrazolium salt into a water-insoluble purple formazan precipitate. The purple formazan crystals are produced by dehydrogenases in active mitochondria in viable cells. Dead cells are unable to perform this reaction. 3(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; thiazol blue (MTT) stock solution (10 µl, 5 mg ml^{-1}) is added to each well. For the stock solution, 5 mg MTT ml^{-1} is dissolved in PBS, passed through a 0.22 µm filter and kept at 4°C for no more than 2 weeks. The parasites are incubated at 25°C for another 8–16 h before the reaction is stopped and the insoluble formazan crystals are solubilized by adding 100 µl 10% SDS in acetate buffer, pH 4.7 and incubated for a further 6–16 h at 37°C. The relative absorbance is determined with a spectrophotometer at 570 nm and a reference wavelength of 630 nm.

◆◆◆◆◆◆ REDIRECTION OF IMMUNE RESPONSES

As discussed earlier in this chapter, a variety of immunological manipulations can drastically change the normal course of progressive disease in BALB/c mice. These changes clearly promote the development of Th1 responses. For a successful redirection, the intervention has to be done early in infection, and these manipulations might interfere with the normally induced pathway of T-cell activation and differentiation. We describe two ways to redirect the non-healing response of BALB/c mice to infections with *L. major*.

Transient Depletion of T-helper Cells *In Vivo*

Rat anti-CD4 mAb GK1.5, isotype IgG2b (Dialynas *et al.*, 1983), is used for the transient depletion of Th cells in an intact mouse. The monoclonal antibodies are purifed by affinity chromatography from cell culture supernatants, filtered sterile and kept at concentrations $\geq 500\,\mu g\,ml^{-1}$ in PBS at 4°C .

Before the purified antibodies are used in an experiment, their potential to deplete Th cells *in vivo* needs to be evaluated. For this purpose, naive mice are injected intraperitoneally with different doses of the purified anti-CD4 mAb, and 48 h later the spleen is removed and the efficiency of this batch of anti-CD4 mAb in the reduction of Th cells is evaluated by flow cytometry. Untreated mice serve as controls. Normally injection of a functional batch of purified anti-CD4 mAb completely eliminates $CD4^+$ T cells below the detection limit 48 h after injection.

In our hands, injection of a total of 400–600 µg purified anti-CD4 mAb per mouse results in the reversal of the non-healing phenotype of BALB/c mice (Fig. 3). These anti-CD4 treated BALB/c mice can resolve the cutaneous lesions, restrict the parasite growth and develop immunity to reinfection (Müller *et al.*, 1991).

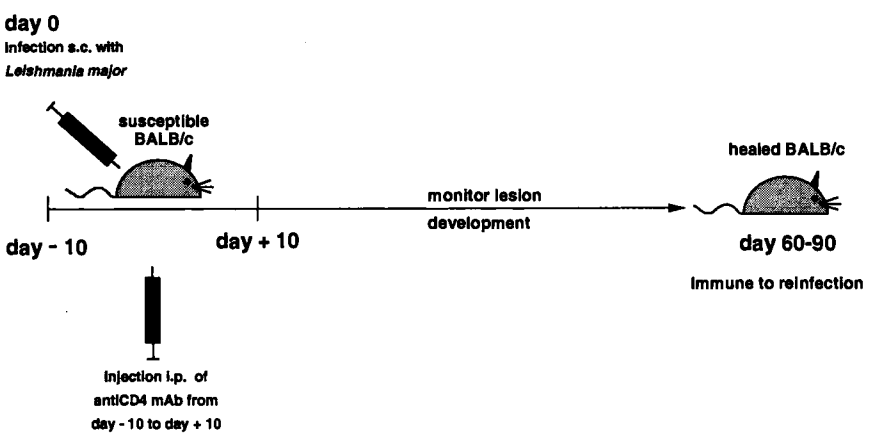

Figure 3. Transient depletion of Th cells *in vivo*.

The anti-CD4 mAb need to be injected in a time span from 10 days before to 10 days after infection to change the non-healing phenotype (Müller *et al.*, 1988). Normally we inject the anti-CD4 mAb at the time of infection or the day before. The antibodies can be injected in a single dose of 600 μg or in two doses of 300 μg in 24 h.

Neutralization of IL-4 *In Vivo*

Similar to the above-described reversal of the non-healing phenotype of BALB/c mice, a single injection of neutralizing anti-IL-4 mAb will also change the immune status of *L. major* infected BALB/c mice. These mice will express a Th1 response, heal the primary infection and acquire immunity to reinfection (Chatelain *et al.*, 1992; Sadick *et al.*,1990). Anti-IL-4 mAb are purified from hybridoma culture supernatants and the most frequently used antibody is 11B11 (rat IgG1) (Ohara and Paul, 1985). The antibodies have to be administered intraperitoneally 2 days before, or at the latest at the time of infection. Injection of anti-IL-4 mAb in the range of 1–10 mg will result in a stable change in the non-healing response of BALB/c mice.

◆◆◆◆◆◆ CONCLUDING REMARKS

In the last decade, impressive progress has been made in our understanding of immune mechanisms involved in leishmanial infections. T cells, and the cytokines they release upon activation, play a critical role in determining the nature of the immune response to infection with *L. major*. Cytokines form a complex network of synergistic and antagonistic interactions which not only induce, but also control, immune responses (Kelso, 1995; Pearce and Reiner, 1995; Xu *et al.*, 1996).

Experimental leishmaniasis in inbred strains of mice is a useful model for studying a variety of basic questions. First, the cellular mechanisms and the cytokine requirements involved in priming and initiation of an immune response can be studied during primary infections (Scott *et al.*, 1989; Liew and O'Donnel, 1993; Reiner and Locksley, 1995). Second, the model can be used to define ways to correct and redirect detrimental responses (Nabors, 1997). Finally, it can be used to gain an understanding of the complex events that enable immune individuals to mount a protective response against reinfection and to characterize the mechanisms and cells operating during the reinfection.

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The SAS® SHPM Program

SAS® is a popular collection of statistical procedures available on almost any operating system and is used world-wide. Thus, the program will be very accessible to both academic and private researchers, requiring only very little knowledge of the software's command syntax.

```
 /*** Limited Dilution Assay (LDA)                                    ***/
 /*** Single-Hit Poisson Model, Minimum Chi-squared Method            ***/
options nocenter ls=78;

proc iml;
fw = 20;
                        /*** Enter data here... ***/
 /* SAS PROC IML is a matrix programming language. Some familiarity
 /*  with it is very helpful but not essential.
 /* The program needs the following information:
 /* The known dose and the number of negatively responding wells
 /*  for each dose level is entered in the matrix assay1. The values
 /*  are entered in pairs (with the dose first and number of negative
 /*  responses second) that correspond to the columns of a matrix.
 /*  A comma separates the pairs into matrix rows. Only the values
 /*  for dose levels greater than 0 and less than the number of wells
 /*  per dose level are needed.
 /* The number of footpads is the variable nfp.
 /* The total amount of tissue in nanograms is the variable tissue.
 /* The number of wells per dose level is the variable nwell.
 /* The p-value for performing hypothesis tests is the variable rejl.
 /* Example: Assume each dose level has 16 wells, 2 footpads =
 /*  165,000,000 ng of tissue, and dose level 16.5 had 1 negative
 /*  response and dose level 1.65 had 13 negative responses. Then
 /*   assay1 = {16.5 1,1.65 13};
 /*   nfp = 2;
 /*   tissue = 1.65 * 10**8;
 /*   nwell = 16;
 /*   rejl = .05;
```

```
 /*  If there had been a third dose level .165 with 14 negative
 /*   responses then assay1 would have appeared as
 /*   assay1 = {16.5 1,1.65 13,.165 14};
 */
assay1 = {      ,      };
nfp =  ;                  /* the number of footpads              */;
tissue =       ;          /* total nanograms of tissue           */;
nwell =    ;             /* number of replicate wells           */;
rejl =   ;                /* p-value to reject hypothesis        */;

 /*** the next 4 lines prevent accidentally using negative well values
  of 0 and nwell since they contribute nothing to the analysis  ***/;
excmax = (loc(assay1[,2] ^= nwell))`;
assay = assay1[excmax,];
exc0 = (loc(assay[,2] ^= 0))`;
assay = assay[exc0,];

 /*** the next 5 lines calculate values needed for computations ***/;
dd = nrow(assay);
l = assay[,1];
r = assay[,2];
n = repeat(nwell,dd,1);
p = r / n;

cnames = {'l' 'r' 'n'}; /*** column labels ***/;

 /*** Assay Validity Test ***/

w1 = (n # p # (log(p))##2) / (1 - p);
w2 = (n # p # l##2) / (1 - p);
w = w1||w2;
x1 = l;
x2 = 1 / l;
xx = x1||x2;
wxx = w # xx;
sumwxx = wxx[+,];
sumw = w[+,];
xbar = sumwxx / sumw;
x = xx - (repeat(xbar,dd,1));
phihat = -(log(p) / l);
yy = log(phihat)||phihat;
wyy = w # yy;
sumwyy = wyy[+,];
ybar = sumwyy / sumw;
y = yy - repeat(ybar,dd,1);
bhat = ((w # x # y)[+,] / (w # x##2)[+,]);
vbhat = abs(((w # y##2)[+,] - (w # x # y)[+,])
        / (dd - (w # x##2)[+,]));
chi = bhat # (w # x # y)[+,];

 /*** Weighted Least Squares Estimates ***/

w = w2;
sumw = w[+,];
x = x2;
y = y[,2];
phiwm = (w # phihat)[+,] / sumw;
vphiwm = 1 / sumw;
phiest = phiwm;
```

```
 /*** Estimate Validity Test ***/
start validest;
 p = exp(-1 * phiest * l);
 w1 = (n # p # (log(p))##2) / (1 - p);
 w2 = (n # p # l##2) / (1 - p);
 w = w1||w2;
 x1 = l;
 x2 = 1 / l;
 xx = x1||x2;
 wxx = w # xx;
 sumwxx = wxx[+,];
 sumw = w[+,];
 xbar = sumwxx / sumw;
 x = xx - (repeat(xbar,dd));
 yy = log(phihat)||phihat;
 wyy = w # yy;
 sumwyy = wyy[+,];
 ybar = sumwyy / sumw;
 y = yy - repeat(ybar,dd,1);
 bhat = ((w # x # y)[+,] / (w # x##2)[+,]);
 vbhat = abs(((w # y##2)[+,] - (w # x # y)[+,])
         / (dd - (w # x##2)[+,]));
 chisl = bhat # (w # x # y)[+,];
 chisl = max(chisl);
finish;

 /*** Newton's Iterative Method ***/
start newton;
 diff = -.000001; /*** convergence value ***/
 phimc = phiwm;
 iter = 0;
 check = 0;
 run chisq;
 do until(check = 1);
  iter = iter + 1;
  run first;
  run second;
  delta = .001 * (jacob / hess);
  converge = phimc - delta;
  phimc = converge;
  check = (delta > diff);
  run chisq;
 end;
finish newton;


 /*** Function to Evaluate ***/
start chisq;
 expon = phimc # l;
 negxpon = exp(-1 * expon);
 negxpon2 = exp(-2 * expon);
 negxpon3 = exp(-3 * expon);
 posxpon = exp(phimc # l);
 denom = n # (1 - negxpon);
finish chisq;

 /*** First Derivative ***/
start first;
 numerf = n # l # negxpon # (2 # r - n) + r##2 # l # (posxpon - 2);
```

```
 jacob = (numerf / (n # (1 - negxpon)##2))[+,];
finish first;


 /*** Second Derivative ***/
start second;
 numers = n # 1##2 # (n - 2 # r) # (negxpon - negxpon3)
          + r##2 # 1##2 # (posxpon - 4 + 7 # negxpon
                           - 4 # negxpon2);
 hess = (numers / (n # (1 - negxpon)##4))[+,];
finish second;


 /*** Mop-up Work ***/
do;
 run validest;
  chiwm = chisl;
  varwm = vbhat;
 run newton;
  phiest = phimc;
 run validest;
reset noname;
 varmc = 2 * 1/hess;
 u95 = phimc + 1.96 * sqrt(varmc);
 l95 = phimc - 1.96 * sqrt(varmc);
 chimc = chisl;
 pv = 1 - probchi(chi,1);
 pvmc = 1 - probchi(chimc,1);
 parasite = tissue/nfp * phimc;
 inv = 1 / phimc;
 print '   MINIMUM CHI-SQUARED ESTIMATION WITH NEWTONS METHOD';
 run banner;
 print '                        ASSAY VALIDITY TEST',
       '                                    Model 1   Model2',
       'chi-squared AVT                     ' chi,
       'p-values of AVT                     ' pv,
       '                        ESTIMATE VALIDITY TEST',
       'estimated frequency                 ' phimc,
       'chi-squared EVT                     ' chimc,
       'p-value of EVT                      ' pvmc,
       'estimated variance                  ' varmc,
       '95% upper confidence level          ' u95,
       '95% lower confidence level          ' l95,
       '1/frequency                         ' inv,
       'estimated parasites per lesion      ' parasite,
       'nanograms of tissue                 ' tissue,
       'number of iterations to converge    ' iter;
start banner;
if all(pv > rejl) then
 print
       '       ********************************************',
       '       ***            CONGRATULATIONS           ***',
       '       ***       THE ASSAY IS VALID BECAUSE     ***',
       '       ***  THE NULL HYPOTHESIS WAS NOT REJECTED  ***',
       '       ***       AT THE 'rejl[format=3.2]
       ' ALPHA LEVEL         ***',
       '       ********************************************';
else
 print
```

```
        '       ********************************************',
        '       ***                SO SORRY                ***',
        '       ***     THE ASSAY IS NOT VALID BECAUSE     ***',
        '       ***    THE NULL HYPOTHESIS WAS REJECTED    ***',
        '       ***      AT THE 'rejl[format=3.2]
        ' ALPHA LEVEL         ***',
        '       ********************************************';
if pvmc > rejl then
 print
        '       ********************************************',
        '       ***             CONGRATULATIONS            ***',
        '       ***     THE ESTIMATE IS VALID BECAUSE      ***',
        '       ***  THE NULL HYPOTHESIS WAS NOT REJECTED  ***',
        '       ***       AT THE 'rejl[format=3.2]
        ' ALPHA LEVEL       ***',
        '       ********************************************';
else
 print
        '       ********************************************',
        '       ***                SO SORRY                ***',
        '       ***    THE ESTIMATE IS NOT VALID BECAUSE   ***',
        '       ***    THE NULL HYPOTHESIS WAS REJECTED    ***',
        '       ***      AT THE 'rejl[format=3.2]
        'ALPHA LEVEL            ***',
        '       ********************************************';
finish banner;
end;

quit;
```

The program is freely available and can be obtained from Kevin Brunson (see List of Suppliers).

List of Suppliers

Endogen
30 Commerce Way
Woburn
MA 081801-1059, USA

Tel.: +1 617 937 0890
Fax: +1 617 937 3096
e-mail: http://www.endogen.com

ELISA minikits.

Southern Biotechnology Associates, Inc.
160A Oxmoor Boulevard
Birmingham
AL 35209, USA

Tel.: +1 205 945 1774
Fax: +1 205 945 8768
e-mail: hhtpp://www.southernbiotech.com

Antibodies.

PharMingen
10975 Torreyana Road
San Diego
CA 92121-111, USA

Tel.: +1 619 677 7737
Fax: +1 619 677 7749
e-mail: http://www.pharmingen.com

Antibodies, cytokines.

Difco Laboratories
P.O. Box 331058
Detroit
MI 48232-7058, USA

Tel.: +1 313 462 8500
Fax: +1 313 462 8517

Nutrient agar.

Bellco Glass, Inc.
P.O. Box B
340 Edrudo Road
Vineland
NJ 08360, USA

Tel.: +1 609 691 1075
Fax: +1 609 691 3247
e-mail: sales@bellcoglass.com

Tenbroeck glass homogenizers.

Corning Costar Corp.
45 Nagog Park
Acton
MA 01720, USA

Tel.: +1 508 635 2200
Fax: +1 508 635 2476
e-mail: http://www.corningcostar.com

Tissue culture, plasticware.

Gibco, Life Technologies, Inc.
P.O. Box 68
Grand Island
NY 14072-0068, USA

Tel.: +1 716 774 6700
FAX: +1 716 774 6694
e-mail: http://www.lifetech.com

Cell culture medium, medium supplements.

Mediatech, Inc.
13884 Park Center Road
Herndon
VA 22071, USA

Tel.: +1 703 471 5955
Fax: +1 703 471 0363

Cell culture medium.

Vector Laboratories, Inc.
30 Ingold Road
Burlingame
CA 94010, USA
Tel.: +1 650 697 3600
Fax: +1 650 697 0339

Peanut agglutinin.

Sigma
P.O. Box 14508
St Louis
MO 63178, USA

Tel.: +1 314 771 5765
Fax: +1 314 771 5757
e-mail: http://www.sigma.sial.com

Chemicals.

Harlan Spraque Dawley, Inc.
P.O. Box 29176
Indianapolis
IN 46229-0176, USA

Tel.: +1 317 894 7521
Fax: +1 317 894 1840

Animals (mice, rabbits).

The Jackson Laboratory
600 Main Street
Bar Harbor
MN 04609-1500, USA

Tel.: +1 207 288 5845
Fax: +1 207 288 3398

Mice.

Kroeplin GmbH
Messzeugfabrik
P.O. Box 12555
D-36881 Schlüchtern
Germany

Tel.: +49 6661 860
Fax: +49 6661 8639

Dial calliper.

Heraeus Bereich Thermotech
P.O. Box 1563
D-63450 Hanau
Germany

Tel.: +49 6181 350
FAX: +49 6181 35880

Teflon for bone marrow macrophage cultures.

SAS Institute, Inc.
SAS Campus Drive
Cary
NC 27513-2414, USA

Tel.: +1 919 677 8000
Fax: +1 919 677 8123

Statistics software.

Kevin Brunson
College of Business Administration,
University of Notre Dame
Notre Dame
IN 46556, USA

Tel.: +1 219 631 9448
Fax: +1 219 631 5255
e-mail: kbrunson@vma.cc.nd.edu

SAS version 6.09, TS 450.

5 Art and Science of DNA Vaccines

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INTRODUCTION

The field of DNA vaccines is an area of active investigation, despite its relative infancy. The inception of the technology can be traced to two papers, one published by Benvenisty and Reshef (1986) and one by Wolff *et al.* (1990), that demonstrated expression of proteins *in situ* after administration of plasmid DNA containing the genes encoding those proteins. It was subsequently shown that vaccination with plasmid DNA using the gene gun could lead to the production of antibody responses against an encoded protein (Tang *et al.*, 1992), and that intramuscular injection of DNA could induce cytotoxic T lymphocytes (CTIs) and protective immunity in a cross-strain influenza challenge model (Ulmer *et al.*, 1993). The application of this technology to the development of various vaccines has been vigorous ever since. To date, the immunogenicity and/or efficacy of DNA vaccines has been demonstrated in animal disease models of viruses, bacteria, parasites, cancer, autoimmunity and allergy (for a review see Donnelly *et al.* (1997a)), and several phase I human clinical trials have been initiated.

The purpose of this chapter is two-fold. First, the basic materials and methods involved in preparing and administering DNA vaccines will be described. Second, some of the recent findings that relate to our understanding of the underlying mechanisms of induction of immune responses will be reviewed in the context of how DNA vaccines may be improved. It is hoped that the following will serve as a guide to

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researchers new to the field, as well as those who wish to modify existing DNA vaccines.

◆◆◆◆◆◆ ART OF DNA VACCINES

Materials

The preparation of *Escherichia coli*-derived plasmid DNA expression vectors utilizes standard molecular biology reagents, such as those described below:

- TE buffer: 10 mM Tris–HCl, 1 mM ethylene diamine tetraacetic acid (EDTA), pH 8.0.
- TEN buffer: 40 mM Tris–HCl, 1 mM EDTA, 150 mM NaCl, pH 7.5.
- Single detergent lysis buffer: 50 mM Tris–HCl, 150 mM NaCl, pH 8.0, 0.02% w/v NaN_3, 1% w/v Nonidet P40, plus the following protease inhibitors – 100 mM phenylmethylsulfonylfluoride (PMSF), 2 μg ml^{-1} aprotinin, 2 μg ml^{-1} leupeptin and 1 μg ml^{-1} pepstatin A.

For more details, see Sambrook *et al.* (1989).

For the administration of DNA vaccines to animals such as mice, ferrets, rabbits or non-human primates, the following materials are needed:

- xylazine (20 mg ml^{-1} stock) (Rompun)
- ketamine (100 mg ml^{-1} stock) (Ketaset)
- atropine (0.4 mg ml^{-1} stock)
- 1-, 3- and 5-ml disposable syringes with 25-gauge needles
- 0.3-ml disposable insulin syringe equipped with a 27-gauge needle
- electric clippers (optional)
- scalpel and sutures (optional)
- ethanol (70%).

Methods

Molecular biology

The gene of interest, or fragment thereof, can be generated by polymerase chain reaction (PCR). In some cases it may be desirable to include only the coding region from the ATG to the termination codon. Design the primers to contain restriction sites useful for cloning, such as BglII or BamHI. A typical cloning procedure is as follows:

1. Separate the PCR fragment from unreacted oligomers and template. Cut the fragment with an excess of enzyme for at least 5 h.
2. Extract in phenol and precipitate in ethanol the DNA, and resuspend in Tris/EDTA (TE), pH 8.

3. Set up a ligation to enzyme-digested vector that has been gel purified and dephosphorylated with calf intestinal alkaline phosphatase, extracted in phenol, precipitated in ethanol and resuspended in TE, pH 8. Use an insert vector molar ratio of 6 : 1 in the ligation reaction.
4. Transform competent *E. coli* cells (e.g. DH5, DH5α) with the ligation reaction and plate on plates containing antibiotic (e.g. L-ampicillin) overnight at 37°C.
5. If the background is high, screen colonies by hybridization of plate-lifts to kinase-labelled PCR primer.
6. Pick several hybridization positive colonies, and grow overnight cultures for miniprep purification.
7. Restriction map to identify clones of proper size and orientation.

Sequence verify at least three clones using primers 30 to 50 bp from the restriction site so that bases within the vector can be read as well as 150 to 200 bases within the gene. In this way, the orientation and quality of the PCR-generated gene can be assessed. To ensure that the final clonal isolate does not contain a low level of contamination with another plasmid, dilute the miniprep DNA 1/1000 in TE (pH 8), and then retransform competent *E. coli*. Isolated colonies (at least three) are grown overnight at 37°C for minipreps and preparation of –70°C cell stocks (add 0.8 ml fresh overnight growth to 0.2 ml sterile 80% glycerol, mix well, and freeze on dry ice). Miniprep DNA is again cut with the appropriate restriction enzymes, visualized on a gel, and a single clonal isolate chosen for use in future experiments.

For large-scale preparation of DNA vaccine vectors grown in *E. coli*, cells should be grown to saturation with vigorous aeration in 500 ml growth medium per 2-l shake flask. Cells are harvested and lysed by a modification of the alkaline, sodium dodecylsulfate (SDS) procedure (Sambrook *et al.*, 1989), except that the volumes for cell lysis and DNA extraction are increased three-fold. DNA is purified by double-banding on CsCl/ethidium bromide gradients, followed by 1-butanol extraction, phenol/chloroform extraction (to remove any trace of endotoxin) and precipitation in ethanol. The concentration and purity of each DNA preparation is determined by reading the optical density (OD) at 260 and 280 nm. The OD_{260}/OD_{280} ratio should be ≥ 1.8.

For characterization of plasmid DNA vectors prior to immunization of animals, expression can be assessed by transient transfection *in vitro* and immunoblot analysis. Cell lines such as RD (human rhabodomyosarcoma cell line) are useful because of their transfection efficiency and their muscle cell lineage. They are grown at 37°C, 5% CO_2, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum, 20 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES), 4 mM L-glutamine, and 100 μg ml^{-1} each of penicillin and streptomycin. Transfection can be accomplished by the calcium phosphate procedure, as described in the kit instructions. Five hours after transfection the cells are glycerol shocked (15% glycerol in phosphate

buffered saline (PBS), pH 7.2) for 2.5 min. Cultures are harvested 72 h after transfection by washing the plates twice with 10 ml of cold PBS, pH 7.2, and then adding 5 ml of cold TEN buffer and scraping. For immunoblot analysis, cell pellets are lysed in single detergent lysis buffer and sonicated on ice (2–15 s bursts). Crude lysates can be transferred and probed with appropriate antibody using standard techniques.

Immunization

There are, as yet, no *in vitro* models for the assessment of how DNA vaccines will perform *in vivo*. A crude estimation of relative vaccine efficacy can be made by comparing relative expression levels of a particular antigen *in vitro*. However, in the final analysis, the effectiveness of DNA vaccines must be tested in animal models. In preparation for animal testing, the following guidelines should be followed. Animals should be in good physical condition and free from stress (e.g. recently shipped animals should be allowed a period of recovery) and housed in an American Association for the Accreditation of Laboratory Animal Care (AAALAC) accredited facility and cared for in accordance with the *Guide for the Care and Use of Laboratory Animals* (1996). For administration of DNA vaccines by injection with a syringe, animals should be anaesthetized. This will prevent pain and injury, and facilitate animal handling. Intraperitoneal injection of a solution containing ketamine and xylazine (50 and 20 $\mu g\ g^{-1}$ bodyweight, respectively) in a total volume of 0.3 ml saline, or inhalation of metophane can be used for mice. Larger animals, such as ferrets or non-human primates, can be anaesthetized using ketamine ($30\ mg\ kg^{-1}$)/xylazine ($2\ mg\ kg^{-1}$)/atropine ($1\ mg\ kg^{-1}$) or ketamine ($10\ mg\ kg^{-1}$), respectively.

For injection of DNA vaccines, the injection site should be swapped with ethanol (70%) to provide sterilization and visualization of the muscle groups. As an added measure to facilitate visualization of small muscles, fur around the injection site can be shaved, or a short incision can be made to permit direct observation of the muscle. In mice, effective immunization has been achieved by injection of the quadriceps, tibialis anterior and gastrocnemius muscles. In addition, other routes of injection, such as intradermal, can be effective (Raz *et al.*, 1994). DNA vaccines appear to be best administered in a simple saline or buffered solution alone. However, certain facilitators may, in some cases, be used to increase the potency of DNA vaccines (Davis *et al.*, 1993a; Vitadello *et al.*, 1994). Several parameters of injection technique have been tested (e.g. number of needle sticks, volume speed), with no significant effect being found (Wolff *et al.*, 1991; Manthorpe *et al.*, 1994). DNA vaccines can also be effectively administered using particle bombardment technology, whereby plasmid DNA is coated onto gold beads and propelled directly into cells of skin tissue (Tang *et al.*, 1992; Fynan *et al.*, 1993). In addition to these parenteral routes of administration, DNA vaccines may also be delivered via mucosal routes, e.g. intranasally (Fynan *et al.*, 1993), or by using live vectors such as *Shigella* (Sizemore *et al.*, 1995).

Other considerations

In the choice of vector to use for DNA vaccine purposes, several parameters should be taken into consideration, such as: (a) high expression, through the use of strong promoter–transcription terminator combinations; (b) lack of eukaryotic origin of replication, to minimize replication of vector and integration into genome of vaccinated animal; and (c) stability of plasmid and ability to amplify to high copy number in *E. coli*, in order to produce high yields of plasmid. The effect of other factors, such as size and physical state of the plasmid, on vaccine efficacy have not been well documented.

The measurement of efficacy of DNA vaccine vectors in animals can be by indirect means, such as expression *in situ* (e.g. through the use of reporter genes such as luciferase), or by direct measurement of immune responses, such as antibodies (both systemic and mucosal), proliferation of and cytokine secretion from antigen-restimulated lymphocytes *in vitro*, and cytolytic activity *in vitro*. In addition, vaccine efficacy can sometimes be assessed in animal challenge models. These immunological parameters are described in detail in other chapters of this book (see Chapters I.2, II.2.3, II.2.4 and II.3.4).

◆◆◆◆◆ SCIENCE OF DNA VACCINES

Some of the most interesting features of DNA vaccination that have yet to be completely elucidated include: (a) uptake of DNA by cells after intramuscular injection, (b) potential consequences of expressing foreign antigens in cells, and (c) mechanisms of antigen presentation. Uptake of DNA by muscle cells after intramuscular injection of DNA is not well understood, but a physiological process involving membrane invaginations has been suggested (Wolff *et al.*, 1992). The safety of DNA vaccines has been intensely scrutinized with respect to integration leading to insertional mutagenesis, autoimmune anti-DNA antibodies and immunological tolerance. So far there has been no indication in animal models of integration (Nichols *et al.*, 1995) or induction of anti-DNA antibodies (J. B. Ulmer and C. M. DeWitt, unpublished observations 1996), and the possibility of induction of neonatal tolerance appears to be antigen-specific (Bot *et al.*, 1996; Mor *et al.*, 1996, Wang *et al.*, 1997). Phase I safety studies in humans are ongoing. Recent work on the presentation of antigens after DNA vaccination has shed some light on the induction of cellular immune responses by this mode of vaccination.

Antigen Presentation

There are several possible scenarios for antigen expression and presentation upon intramuscular injection of DNA encoding an antigen. First, antigen expression by muscle cells could lead to direct induction of T-cell responses, i.e. myocytes act as antigen-presenting cells (APCs). Expression of DNA-encoded protein by muscle cells does occur, as has been

documented for reporter genes (Wolff *et al.*, 1990) and antigens (Davis *et al.*, 1993b; J. B. Ulmer and C. M. DeWitt, unpublished observations 1996), but the lack of co-stimulatory molecules on muscle cells make it unlikely that T-cell priming could occur by this means. Secondly, DNA could also transfect APCs after intramuscular injection, which could be an effective way to prime naive T cells for antigen-specific responses. This could involve APCs in the muscle compartment or at distal sites to which plasmid DNA has migrated. Finally, DNA vaccination could result in expression of antigen by muscle cells followed by transfer of antigen to an APC capable of presenting exogenous antigen to T cells (i.e. by cross-priming; see Bevan (1995)). So far, the results with DNA vaccine studies support the hypothesis that antigen is transferred from muscle cells to bone-marrow derived APCs for induction of CTL. The data are as follows. Upon transplantation of stably transfected myoblasts expressing influenza nucleoprotein (NP) into syngeneic or F1 hybrid mice, NP-specific CTL and cross-strain protection were induced (Ulmer *et al.*, 1996a). Therefore, production of antigen by muscles alone is sufficient to induce a CTL response, and transfection of APCs after DNA vaccination is not required. Furthermore, after transplantation of myoblasts into F1 hybrid mice at a site where no fusion with host myocytes could occur (i.e. the peritoneal cavity), CTL restricted by both host major histocompatibility complex (MHC) haplotypes were induced, indicating that transfer of antigen from the implanted myoblasts to a host cell can occur (Ulmer *et al.*, 1996a). This hypothesis is supported by adoptive transfer of APCs into *scid* mice, where it was shown that CTL responses after DNA vaccination could be facilitated by APCs, even when transfer occurred several weeks after DNA vaccination (Doe *et al.*, 1996). At this time after DNA vaccination, the possibility of transfection of the implanted APCs with the previously injected DNA is remote. Hence, transfer of antigen from transfected host cells to donor APCs has probably occurred. The requirement for APCs in the induction of CTL after DNA vaccination was demonstrated using parental → F1 bone marrow chimeric (BMC) mice (Corr *et al.*, 1996; Fu *et al.*, 1997). DNA vaccination of BMC mice clearly indicated that muscle cells were not acting as APCs, but rather cells derived from the bone marrow were responsible for induction of CTL. Furthermore, myoblast transplantation into BMC mice demonstrated that bone marrow-derived APCs were required for induction of CTL, even when the expression of antigen was confined to muscle cells (Fu *et al.*, 1997). Taken together, these observations strongly implicate antigen transfer from transfected muscle cells to APCs as a mechanism of induction of CTL after DNA vaccination. Interestingly, antigen transfer may be a more generalized phenomenon, as bone marrow-derived APCs were also shown to be required for CTL induction in a tumour model (Huang *et al.*, 1994).

Potential Means for Improvement

There are several areas in which increases in DNA vaccine potency can potentially be achieved, such as: modifications to the vector (e.g. expression

levels); use of delivery vehicles that could protect DNA from nuclease digestion, facilitate cellular uptake or target specific cells; and adjuvants that could enhance or modulate immune responses against the expressed protein (for a review, see Ulmer *et al.* (1996b)). In this chapter we focus on two specific areas – immunomodulatory DNA sequences and co-expression of cytokines – since recent developments have been made in these areas. Moreover, direct modifications to the vector (e.g. incorporation of CpG motifs of dicistronic constructs encoding cytokine genes) or use of DNA vaccines in combination with CpG oliogonucleotides or cytokine-encoding plasmids are two of the simplest approaches that one could take toward DNA vaccine improvement.

The immunomodulatory effects of specific bacterial DNA sequences have been known for several years (Messina *et al.*, 1991; Yamamoto *et al.*, 1992), but have only been studied in detail recently. It has been shown that an unmethylated oligonucleotide motif consisting of purine–purine–C–G–pyrimidine–pyrimidine is a very potent stimulator of natural killer (NK) cells and lymphocytes *in vitro* (Klinman *et al.*, 1996), and can have immunostimulatory effects *in vivo* (Krieg *et al.*, 1995). The potential adjuvant effects of *E. coli* derived plasmid DNA vectors was suggested in two preliminary observations. First, co-administration in mice of NP protein with a plasmid DNA vector not encoding a protein resulted in an alteration of the isotype profile of antibodies toward a predominance of immunoglobulin G2a (IgG2a) induced against NP (Donnelly *et al.*, 1997b). Secondly, the immune responses against influenza haemagglutinin using a haemagglutinin DNA vector were increased if additional non-coding plasmid was included in the inoculum (Donnelly *et al.*, 1997a). The potential use of immunostimulatory DNA sequences directly in DNA vaccines was suggested by Sato *et al.* (1996) who incorporated a specific motif (AACGTT) into a DNA vaccine vector encoding β-galactosidase resulting in substantial increases in cellular immune responses against the encoded antigen. However, there is much to be evaluated before the utility of this approach can be ascertained, such as: (a) general applicability to other antigens and vectors, (b) positional effects of CpG motifs, (c) the stimulatory effects (or lack thereof) of the many other Pu-Pu-C-G-Py-Py motifs present in plasmid vectors, and (d) potential induction of pathogenic anti-DNA antibodies. Nevertheless, it is an attractive approach worthy of attention due to its minimal effect on the complexity of vaccine formulation.

Expression of cytokines is another means of modulating or enhancing immune responses against antigens encoded by DNA vaccines, and some success has been reported. Co-expression of granulocyte–macrophage colony stimulating factor (GM-CSF) in mice has resulted in enhanced antibody responses against rabies virus glycoprotein (Xiang and Ertl, 1995), carcino-embryonic antigen (Conry *et al.*, 1996) and human immunodeficiency virus (HIV env) envelope protein (Kim *et al.*, 1997). Vaccination of mice with DNA plasmids encoding the hepatitis B surface antigen (HBsAg) and IL-2, either as a fusion protein or as discrete gene products, were effective at increasing the antibody and cellular immune responses to HBsAg (Chow *et al.*, 1997). Modulation of the immune

responses against HIV env were observed upon co-administration of HIV DNA with an IL-12 DNA plasmid, where reduced antibody titres and increased cellular immune responses against HIV env were induced compared to vaccination with HIV DNA alone (Kim *et al.*, 1997). A DNA plasmid encoding the β-chemokine TCA3 was recently shown to have adjuvant activity that resulted in an augmented cellular immune response induced by HIV env DNA (Tsuji *et al.*, 1997). Finally, in an animal model of B-cell lymphoma vaccination of mice with a DNA encoding a fusion protein consisting of a single chain Fv and a nine amino acid peptide from interleukin 1β (IL-1β) resulted in protection from tumour challenge (Hakim *et al.*, 1996). Taken together, these observations suggest that modulation or enhancement of specific immune responses induced by DNA vaccines can be achieved. However, the potential use of this approach in humans may be limited, in the first instance, to therapeutic applications, and prophylactic use may require the application of regulatable promoters to prevent the uncontrolled expression of cytokines in healthy individuals.

◆◆◆◆◆ SUMMARY

The prospects for DNA vaccination are encouraging, as progress towards a greater understanding of the basic mechanisms and safety implications continues. Advances in how antigens are presented to the immune system and rational approaches to how the potency of DNA vaccines can potentially be improved will guide our strategies toward developing DNA vaccines for human use.

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List of Suppliers

One of the greatest assets of the DNA vaccine technology is its simplicity. Standard materials available from many different commercial sources can be used to prepare, administer and test DNA vaccines. One exception,

however, is if a gene gun (rather than a syringe) is used to deliver DNA. This devise (Biolistic Particle Delivery System) and the materials required for vaccine preparation can be obtained commercially from:

Bio-Rad Laboratories
2000 Alfred Nobel Drive
Hercules
CA 94547, USA

Tel.: +1 510 741 1000
Fax: +1 510 741 5800

6 Preparation and Use of Adjuvants

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INTRODUCTION

The immunogenicity of an antigen–adjuvant formulation, in terms of humoral and cellular immune responses, is dependent on a range of different factors. Among these are the physical characteristics of the antigen, in particular its size, charge and degree of glycosylation, the antigenicity and innate adjuvanticity, and the choice of adjuvant. Most of these factors regarding the physical and biological characteristics of the antigen cannot easily be altered, but the adjuvant part can be selected to suit a particular protein and to modulate the immune responses in a desired direction, for example with an emphasis on an antibody response dominated by certain isotypes or subclasses, or a need for cellular effector mechanisms such as cytotoxic T cells.

In the literature there are numerous examples of different antigens and their performance with various adjuvants, review articles and book chapters on adjuvants and adjuvant characteristics. However, despite all this information, it is difficult to predict the immunological outcome of a previously untested adjuvant–antigen combination. Adjuvant research is far less empirical today than 20 years ago. Many more of the mechanisms behind adjuvant activity are known, helping us to make rational decisions in our choice of adjuvant formulation for a certain antigen preparation.

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This chapter focuses on the interactions between antigens and a few commonly used adjuvants, identifying some key factors affecting the success of the antigen–adjuvant formulation. It is not possible to cover here all the types of adjuvants available today, but a selected number of adjuvants and adjuvant formulations will serve as examples of the parameters involved.

◆◆◆◆◆◆ ADJUVANT ACTIVITY AND ANTIGEN–ADJUVANT FORMULATION

The purpose of an adjuvant is simply to increase the immunogenicity of an antigen and to modulate the immune response to the antigen in a desired direction. Though we cannot generalize, considering the mode of action of the hundreds of adjuvant formulations available today (Cox and Coulter, 1992, 1997; Vogel and Powell, 1995) there are three major areas in which adjuvants exert their adjuvant activities or, alternatively, where antigens require help to increase their immunogenicity (Morein *et al.*, 1996). These areas are:

- *Physical presentation of the antigen.* This involves the physical appearance and the antigenicity of the antigen in an adjuvant formulation. Factors included here are stabilization and exposure of native conformational epitopes in the antigen, adjuvant-mediated formulation of the antigen into small soluble particles or aggregates, and other mechanisms bringing the antigen into an organized multimeric formation and hence increasing the surface area of the antigen.
- *Antigen and adjuvant uptake and distribution (targeting).* This covers a whole range of activities, including slow release of antigen from a depot at the injection site, the initiation of the immune response by attracting appropriate antigen-presenting cells, and other activities leading to increased antigen uptake and transport of antigen to relevant lymphatic organs. A subset of these activities covers the intracellular handling of antigens, especially their proteolytic processing and association with major histocompatibility complex (MHC) class I or II molecules.
- *Immune potentiation and modulation.* These include activities that regulate both quantitative and qualitative aspects of the ensuing immune responses, generally as a result of modulation of cytokine networks. This will usually result in expansion of T-cell clones with different profiles of cytokine production.

Very few adjuvants exert strong activities in all three areas, and for poor antigens that require extensive adjuvant help a combination of adjuvants may be required for appropriate immune stimulation.

The term 'adjuvant' or 'adjuvant formulation' covers activities within one or more of the three areas mentioned above and possibly other mechanisms leading to increased immunogenicity of an antigen. Allison and Byars (1990) introduced some structure into the terminology by defining an adjuvant as an agent that augments specific immune stimulation to antigens, a vehicle as the substance used for the delivery of the antigen, and an adjuvant formulation as the combination of adjuvants in a suitable

vehicle. However, since vehicles also may exert adjuvant activities, especially in the area of antigen presentation, the concept of adjuvant activity remains unclear.

In order to discuss the practical aspects of adjuvants, vehicles and adjuvant formulations, this chapter will not strictly follow this definition, but rather uses the term 'adjuvant' in a broader sense to mean to help (increase immunogenicity), as from its Latin origin *adjuvare* (to help). The only clear distinction that will be made is that between particulate and non-particulate adjuvants (Cox and Coulter, 1992).

◆◆◆◆◆◆ ANTIGENS

The physical and biological variability of antigens forms a continuum. In practice it is useful to make the following categorization.

Particulate Antigens

Among the particulate antigens we find whole cells and micro-organisms or various sized parts thereof. Smaller particles (of the order of nanometres) are protein micelles (or rosettes) and other small aggregates spontaneously formed in aqueous solution by hydrophobic and amphipathic antigens. Particulate antigens are generally good immunogens and it is comparatively easy to increase their immunogenicity further by the addition of an immunomodulatory adjuvant.

Monomeric Antigens

Monomeric antigens are proteins that do not spontaneously form particles or aggregates in solution. These antigens are generally poor immunogens and require help from potent adjuvants. Since the antigens themselves do not form particles, these antigens often perform well when administered with particulate adjuvants. Unfortunately, most recombinant-DNA products belong to this group of antigens, and this has so far limited their use in vaccines.

Innate Immunogenicity: Antigen-induced Immunomodulation

Many antigens, especially those derived from micro-organisms, have immunomodulatory effects. The immunomodulation can be both positive and negative, and cannot easily be overcome by addition of adjuvants. For example, the flagellar fraction of *Trypanosoma cruzi* contains an antigen (Ag 123) that has immunosuppressive activity (Hansen *et al.*, 1997). This antigen possibly prevents the development or the recall of memory cells secreting γ-interferon (IFN-γ) (Hansen *et al.*, 1996).

The fact that some antigens are immunogenic at 100-fold smaller doses than others and that each antigen–adjuvant combination has its optimum of both adjuvant and antigen dose should be taken into consideration.

Antigen Dose Modulation

It has recently been shown that not only the physical and biological activities of an antigen influence its immunogenicity, but also the dose of the antigen may modulate the immune responses. For example, it has been shown that a medium dose of peptide antigen induced a T-helper 0/T-helper 1 cell (Th0/Th1) type response, while high and low doses of the same peptide induced predominantly Th2 responses (Mosmann and Coffman, 1989; Bretscher *et al.*, 1992; Hosken *et al.*, 1995). It was also recently shown that neonatal tolerance may not be an intrinsic property of the newborn immune system. Instead the dose of antigen had to be balanced against the low number of virgin T cells present in the newborn spleen, and consequently the unresponsiveness of newborn mice could be avoided simply by lowering the antigen dose (Forsthuber *et al.*, 1996; Ridge *et al.*, 1996; Sarzotti *et al.*, 1996).

◆◆◆◆◆◆ PARTICULATE ADJUVANTS

Particulate adjuvants are adjuvants that are capable of arranging antigens into particle-like organizations, small aggregates, droplets or precipitates, where such activity is an important component of their adjuvant activity (Cox and Coulter, 1992). It is among the particulate adjuvants we find the most frequently used adjuvants, such as Freund's complete and incomplete adjuvant and the aluminium salt gels.

Aluminium Salts

The history of aluminium adjuvants begins early this century with the observation that alum precipitation significantly increased the immunogenicity of toxoids (Glenny *et al.*, 1926). Alum-precipitated antigens were prepared by mixing antigens in solution with potassium alum to form protein aluminates, a product that could be highly heterogeneous depending on which anions (bicarbonate, sulphate or phosphate) were present in the mixture. This heterogeneity was later overcome by the use of preformed aluminium hydroxide and aluminium phosphate gels to which antigens were adsorbed. However, preformed gels also differ in terms of physico-chemical characteristics, stability and protein adsorption. To permit comparisons between research activities, an international workshop elected to use one good commercial preparation as the research standard (Stewart-Tull, 1989). Although aluminium adjuvants have immunostimulatory activities such as attraction of eosinophils to the injection site (Walls, 1977) and complement activation (Ramanathan *et al.*, 1979), most evidence indicates the absorption of antigens to the gel as the

key adjuvant activity. The mechanisms responsible for adsorption of antigens include intramolecular forces such as electrostatic attraction, hydrophilic and hydrophobic interactions, hydrogen bonds and van der Waals forces (Seeber *et al.*, 1991).

The antigen is adsorbed to the gel by incubation (slow agitation) at a preselected pH for a few hours or overnight. The pH optimum for adsorption varies between antigens. In a mixture of antigens some may bind better than other, and to get a reproducible and reliable binding different pH values for both the hydroxide and phosphate gels need to be tested. For example, Seeber *et al.* (1991) showed that proteins with an acid isoelectric point adsorb better to aluminium hydroxide and that proteins with an alkaline isoelectric point adsorb better to aluminium phosphate.

The stability of the antigen is also of vital importance. Some antigens are highly sensitive to pH alterations and the final pH chosen may very well be a compromise between antigen stability and antigen adsorption to the gel. The degree of antigen adsorption to the gel can readily be tested. After incubation of the antigen with the gel at different pH, the gel is spun in a simple low speed centrifuge and the clear supernatant is tested for presence of antigen by enzyme-linked immunosorbent assay (ELISA), electrophoresis, bioassay or any other suitable technique.

Type of immune modulation

Adsorption of antigens to $Al(OH)_3$ increases the efficiency of antigen presentation to antigen-presenting cells and upregulates the latter as measured by an increased interleukin-1 (IL-1) production (Mannhalter *et al.*, 1985). $Al(OH)_3$ activates complement (Ramanathan *et al.*, 1979), improves antigen trapping in lymph nodes, and acts as a short-term (1–2 weeks) depot for antigen release. Thus the antigen is presented in a 'particulate' manner and the rate of antigen targeting is increased (Mannhalter *et al.*, 1985). These properties of $Al(OH)_3$ are probably the major reasons why it works so well with small soluble antigens such as toxoids. Experiments done with $Al(OH)_3$ and different antigens suggest that $Al(OH)_3$ is superior with poorly immunogenic antigens, but of less value in potentiating the immunogenicity of strong antigens (Bomford, 1984). The resulting immune modulation afforded by $Al(OH)_3$ is moderate and mainly affects the antibody response, characterized by an increased production of IL-4 leading to a Th2-type antibody response dominated by murine IgG1 antibodies (IgG2 in humans) but poor cell-mediated responses (CMI) (Bomford, 1980a). Whether the Th2-type response (Mosmann and Coffman, 1989; Hu and Kitagawa, 1990) is an immunomodulatory effect of $Al(OH)_3$ or merely a potentiation of the response generated by the antigen itself is not clear, since monomeric, poorly immunogenic antigens will themselves induce a Th2-type response. However, the induction of IgE, at least in rodents (Hamamoka *et al.*, 1973; Uede *et al.*, 1982; Uede and Ishizaka, 1982), may imply the former. The value of $Al(OH)_3$ in the induction of secondary responses is often low after a strong priming (Hu and Kitagawa, 1990).

Dosing

The amount of $Al(OH)_3$ gel used for injection has to be tested for each antigen and species, but the concentration normally varies in the range 15–40% v/v. No more than 1.25 mg aluminium is allowed in one dose of human vaccine.

Oil Emulsions

Water-in-oil emulsions

The aluminium salts adsorb antigen to a gel, while in oil adjuvants the antigens are in aqueous-phase droplets in water-in-oil (w/o) emulsions, best exemplified by Freund's complete (FCA) and incomplete (FIA) adjuvants. A w/o emulsion is an even dispersion of microdroplets of water in a continuous phase of oil. As a lot of energy is required for its production, the resulting emulsion becomes unstable unless an emulsifier (an agent consisting of polar and non-polar regions) is added to reduce the interfacial tension.

The most common oils used in w/o adjuvants are light mineral oils such as Drakeol 6 VR, Bayol F and Marcol 52. These are complicated mixtures, but usually contain varying proportions of paraffin and naphthene derivatives (Dalsgaard *et al.*, 1990). Arlacel A (mannide monooleate) was one of the first emulsifiers commonly used, and is still in use in a more purified form (Arlacel A Special, ICI, Montanide 80, Seppic). Objection to the use of mineral oils for vaccines for food animals and, in some countries, all animals, have led to the search for metabolizable alternatives. Vegetable oils such as peanut and sesame oils are reported to be useful, but are less efficient than FIA (Hilleman, 1966; Fukumi, 1967; Kimura *et al.*, 1978; Brugh *et al.*, 1983). Squalene, an intermediate in the biosynthesis of cholesterol and its hydrogenated form, squalane, are preferred options.

A report that Arlacel A is carcinogenic in Swiss Webster mice and co-carcinogenic in all strains of mice tested (Murray *et al.*, 1972) increased the importance of alternative biodegradable emulsifiers. A blend of Tween 85 and Span 85 (Bokhout *et al.*, 1981) is reported to form stable emulsions with higher adjuvant activity than FIA (Ott *et al.*, 1995). The non-ionic Pluronic 122 (see below) has been used as an emulsifier for a range of fatty acid esters, with good results (Bomford, 1981).

Type of immune modulation

Water-in-oil formulations are generally efficient adjuvants and their main adjuvant effects rely on their ability to target the antigen to the immune system. The antigen must be located in microdroplets of water which are in the oil phase, but such an emulsion can be formed with almost any antigen, regardless of its size, charge or other physical characteristics, provided it is water soluble. However, it is argued that antigens may denature during the emulsification process (Kenney *et al.*, 1989), and w/o emulsions are possibly less well suited for antigens where preservation of

conformational epitopes is vital. Water-in-oil formulations are highly efficient for inducing T-cell responses and antibody responses to linear B-cell epitopes. After injection, the antigen is continuously released from a depot formed at the injection site, attracting mononuclear cells. However, the usefulness of this depot is debated, since excision of the depot 6–8 weeks (Lascelles *et al.*, 1989) after administration failed to affect the immune responses. Like $Al(OH)_3$, w/o emulsions without additional immunomodulators such as mycobacteria in the case of FCA, induce mouse antibodies predominantly of the IgG1 isotype, and do not stimulate delayed-type hypersensitivity (White, 1976).

Oil-in-water emulsions

Oil-in-water emulsions consist of microdroplets of oil in a continuous phase of water, and were produced in attempts to prepare emulsions of reduced viscosity compared to w/o emulsions (Meyer *et al.*, 1974). In addition, emulsions with reduced amounts of oil would be expected to be less reactogenic. The oil phase of oil-in-water (o/w) emulsions is typically 1–5%, compared to 25–50% in w/o emulsions.

Type of immune modulation

Oil-in-water emulsions are generally less potent than w/o formulations, at least with regard to the duration of the immune response (Dalsgaard *et al.*, 1990). They are generally well tolerated and have a viscosity which is low compared to w/o formulations. Also, in o/w emulsions, hydrophobic and amphipathic antigens are presented in an organized particulate form (Dalsgaard, 1987). However, it is generally believed that addition of surface-active molecules such as non-ionic block co-polymers (Allison and Byars, 1986), sorbitan or glycerol trioleate (Woodard, 1989) or trehalose dimycolate (TDM) (McLaughlin *et al.*, 1978; Hunter *et al.*, 1981; Lemaire *et al.*, 1986) is essential for optimum presentation of most antigens. Oil-in-water emulsions are generally not considered to have immunomodulatory activity, but they offer a suitable vehicle for a range of immunomodulatory substances.

Water/oil/water emulsions

Water/oil/water emulsions (i.e. a w/o emulsion re-emulsified in water) have also been made in attempts to reduce the viscosity of w/o emulsions (Hunter *et al.*, 1995). These are technically difficult to prepare and are less stable than w/o and o/w emulsions.

Non-ionic Block Polymers

The finding that many adjuvants are surface-active molecules led Hunter and co-workers to synthesize and study a new group of surface-active molecules with an apparent adjuvant effect – the non-ionic block

polymers (NBPs) (Hunter *et al.*, 1981; Hunter and Bennett, 1984, 1986). NBPs are co-polymers of polyoxyethylene and polyoxypropylene of various chain length and different hydrophilic/lipophilic balance (HLB). The adjuvant active NBPs tend to have a lower HLB, i.e. they are more lipophilic. Many of these polymers also act as emulsifiers, forming stable w/o, o/w and w/o/w emulsions.

Oil-in-water co-polymer adjuvants have been reported to have induced potent immune responses and protection in many studies (Byars and Allison, 1987; Millet *et al.*, 1992). The co-polymers used in these studies have one severe disadvantage; they are insoluble at room temperature despite being soluble when refrigerated (Hunter *et al.*, 1995). Consequently, the emulsion is destabilized when refrigerated. This problem can be solved by microfluidization of the emulsion; however, even then the emulsions are less effective adjuvants than classical o/w emulsions (Hunter *et al.*, 1981).

The largest co-polymers are particularly effective in stabilizing w/o emulsions. For example, water-in-squalene emulsions containing up to 90% water were stable at room temperature for months, and can even be frozen and thawed (Hunter *et al.*, 1995).

Large block polymers can also stabilize w/o/w emulsions. Such double emulsions have been used with Simian immunodeficiency virus (SIV) (Hunter *et al.*, 1995) and malaria (Millet *et al.*, 1992) in monkeys with good efficacy, but with some severe local reactions.

The new larger co-polymers also form stable microparticulate suspensions in saline without oil. The efficacy of these preparations is largely dependent on the physical properties of the antigens, since antigens that fail to bind to the polymer give poorer responses (Hunter *et al.*, 1995), while antigens that bind become highly immunogenic.

TiterMax and TiterMax Gold (CytRx Corporation)

TiterMax emulsions consist of 80% saline in squalene containing block copolymer CRL-8941, sorbitan monooleate and co-polymer-coated silica particles (Hunter *et al.*, 1995).

In January 1997, CytRx released a new and improved w/o adjuvant, called TiterMax Gold. This formulation contains a new block co-polymer, CRL-8300.

Type of immune modulation

The precise mode of action of this class of adjuvant formulation is unknown, but the binding of antigen to the polymer seems to be essential, since co-polymers with low antigen binding capacity have a low adjuvant activity. The co-polymers also augment the expression of class II major histocompatibility antigen by macrophages (Howerton *et al.*, 1990) and it is likely that the major activities exerted by the block co-polymers relate to a favourable physical presentation of the antigen in a condensed three-dimensional matrix in a milieu of activated antigen-presenting cells (Hunter *et al.*, 1994).

TiterMax formulations are generally immunogenic also with monomeric-type antigens. The primary antibody response is often prominent, reaching a plateau after about 6–8 weeks, after which the level of antibody slowly declines. The value of a booster dose is, however, controversial. In some experiments it seems to have had no or only a very low value, and sometimes it gives a marginal effect. A booster dose with the antigen alone has been shown to be more efficient than a booster with TiterMax emulsion (Bennett *et al.*, 1992).

Of greater value than a booster is to divide the dose and increase the number of injection sites. A four-site administration procedure will induce IgG titres that are almost 10 times higher than those obtained with the same amount of vaccine divided into two sites (Bennett *et al.*, 1992). Mice dosed with antigens in TiterMax emulsion respond with comparable levels of IgG1 and IgG2b, variable levels of IgG2a and low levels of IgG3 (Kalish *et al.*, 1991). TiterMax emulsions also effectively induce T-cell responses, including $CD8^+$ class I restricted cytotoxic T lymphocytes (Feltkamp *et al.*, 1993).

Dosing

- *TiterMax.* Water/TiterMax ratios of 90:10 to 50:50 can be used, although 50:50 is generally optimum. Division of the vaccine dose at multiple injection sites is beneficial. The dose span from mouse to cow/horse is 40–1000 µl. Dose suggestions for a number of animal species are available on the TiterMax home page (http://www.titermax.com/i_tech.htm).
- *TiterMax Gold.* Good immune responses have been achieved with a water/TiterMax Gold ratio of 50:50. Estimated dose recommendations based on the results with TitreMax Gold are given on the TiterMax home page (http://www.titermax.com/i_gold.htm). In general, 100–400 µl of vaccine in 1–4 does sites is suitable for most animal species.

Liposomes

Lipid vesicles or liposomes are vehicles for vaccine adjuvant formulations that have attracted the interest of many groups due to their versatility. Liposomes with physically diverse properties can be made by altering the composition of the lipids and the method used for production (Alving, 1992). Liposomes range in size from 20 nm up to more than 10 µm, and they can be uni- or multilamellar, rigid or fusogenic, charged or non-charged. Hydrophilic antigens can be contained in the interior or between the lipid bilayers, while hydrophobic or amphipathic antigens can be integrated in the lipid membrane during the preparation. Due to the great number of possible variations of both the composition of liposomes and their interaction with different antigens, it is difficult to generalize their effects. It is clear that liposomes are potent in presenting antigens, especially hydrophobic or amphipathic antigens presented on the liposomal surface. Liposomes by themselves are not generally regarded as particles that efficiently enhance or modulate the immune response, and require

supplementation with immunomodulators. However, a recent paper (Phillips *et al.*, 1996) indicates that the phospholipid composition influences the antibody response to encapsulated antigens. Almost every possible combination of liposomes and other adjuvants has been tested (Alving, 1991; Kersten and Crommelin, 1995).

A recently described form of antigen presentation related to liposomes are the so-called protein cochleate formulations. These are protein–lipid (cholesterol/phosphatidyl serine)–calcium (Ca^{2+}) precipitates consisting of a large continuous solid lipid bilayer sheet rolled up in a 'jelly roll' fashion (Gould-Fogerite *et al.*, 1994). Amphipathic or hydrophobic antigens can be integrated into the lipid bilayer of the cochleates. The presence of calcium maintains the cochleates in their rolled-up form, and removal of the calcium by diffusion or addition of a chelating agent such as ethylene diamine tetraacetic acid (EDTA) allows the cochleate to unroll and form large, mainly unilaminar, liposomes.

Trehalose Dimycolate

Trehalose-6,6'-dimycolate (TDM) is a high-molecular-weight glycolipid isolated from mycobacteria (Bloch, 1950; Noll *et al.*, 1956). TDM is an amphipathic surface-active molecule frequently employed in o/w emulsions in the presence of Tween 80 (Lemaire *et al.*, 1986). The orientation of TDM in o/w emulsions promotes retention of soluble antigens (McLaughlin *et al.*, 1978) and is able to concentrate soluble antigens on the surface of the oil droplets. The efficacy of TDM as an adjuvant in emulsions depends on the physical characteristics of the emulsion. The most potent emulsions of TDM comprise small oil droplets with concomitant large surface areas (Rudbach *et al.*, 1995).

Cell-wall Skeleton

The mycobacterium cell-wall skeleton (CWS) is defined as the material remaining after nucleic acid, protein and free fatty acid have been removed from the cell wall. The CWS is a particulate complex consisting of peptidoglycan, a polymerized form of muramyl dipeptide (MDP), arabinogalactane and mycolic acid (Rudbach *et al.*, 1995). When formulated in o/w emulsions, the CWS tends to coat the surface of the oil droplets (McLaughlin *et al.*, 1978).

The immunostimulatory effect of the CWS is probably mediated by several parts of the complex, since both peptidoglycan and mycolic acid are immunostimulatory.

The CWS is one of the constituents in Ribi adjuvant systems (see below).

◆◆◆◆◆◆ NON-PARTICULATE ADJUVANTS

Saponins

Data on saponin derivation are often not available. However, most adjuvant-active saponin preparations are probably derived from the South American soap tree *Quillaja saponaria* Molina. Saponins from other plants may also be adjuvant active, and saponins in general are often mentioned as the active component in medicinal plants (Campbell, 1995).

The adjuvant activity of *Quillaja* saponins was recognized in the early literature on adjuvants (Ramon, 1926; Thibault and Richou, 1936) and their commercial use in vaccines dates back to 1951 (Espinet, 1951). Since the pioneer work of Dalsgaard (1974, 1978), much effort has been directed at the separation of *Quillaja* saponin extracts in order to identify which components are adjuvant active and which components are responsible for undesired effects such as toxicity. The goal for this activity has been to find a component or a fraction of *Quillaja* saponin that is well characterized and suitable for use in human vaccines. Currently, there are two potential candidates: QS-21 (Kensil *et al.*, 1991) and Iscoprep 703 (Barr and Mitchell, 1996; Vogel and Powell, 1995). QS-21 is used in simple addition to an antigen in solution or mixed with $Al(OH)_3$ while Iscoprep 703 is intended for production of iscoms or iscom-matrix (see below). QS-21 is a substantially pure saponin component, whereas Iscoprep 703 consists of two fractions of *Quillaja* saponins. Iscoprep 703 is designed not only for optimum adjuvant activity with minimum toxicity, but also to efficiently form iscoms with a range of different antigens and lipids (see below).

Type of immune modulation

In general, saponins induce potent antibody and cell-mediated immune responses, especially with antigens in cell membranes (Bomford, 1980a,b; Scott *et al.*, 1984) and other particulate antigens. Monomeric or small antigens, i.e. weak antigens, often perform poorly when adjuvanted with saponins (Bomford, 1984), most likely because saponins as such do not provide good physical antigen presentation. However, saponin used in iscoms (see below) or combined with $Al(OH)_3$ (Dalsgaard, 1978; Ma *et al.*, 1994) or liposomes (Lipford *et al.*, 1994), to confer a good physical presentation of antigen, often yield very efficient adjuvant formulations, inducing both cellular and humoral responses. Scott *et al.* (1984) noted an acute local inflammation after subcutaneous injection of unfractionated *Quillaja* saponins. Although antigen was retained at the injection site, increased amounts also reached the spleen. The local inflammation was probably caused by cell and/or tissue damage due to saponin binding to cell-membrane cholesterol. Both the inflammation and the splenic localization was blocked by added liposomal cholesterol, suggesting that these effects were mediated through the local inflammation. In contrast, iscoms and iscom-matrix (see below) comprise *Quillaja* saponins bound to cholesterol, and hence a local inflammation is less likely to occur and the antigen is rapidly removed from the injection site and focused to the spleen

(Watson *et al.*, 1989; Lövgren-Bengtsson and Sjölander, 1996; Sjölander *et al.*, 1996a, 1997a,b). The immune potentiation of *Quillaja* saponins is probably mediated by several mechanisms and influenced not only by the antigen but also by the antigen presentation form.

There is conflict about which subclasses of IgG are enhanced (Scott *et al.*, 1984; Kenney *et al.*, 1989; Karagouni and Hadjipetrou-Kourounakis, 1990; Kensil *et al.*, 1991). However oral ingestion of saponins resulted in a non-specific resistance to intracerebral challenge with rabies virus (Chavali and Campbell, 1987), suggesting a stimulation of IL-2 and IFN-γ. This is supported by the results of Heath *et al.* (1991), which showed that saponin seems to mimic the effects of IFN-γ, suggesting that one property of saponin is release of IFN-γ.

Dosing

As with most adjuvants, the dose of saponin should be adjusted to the antigen and the animal species. Attention must also be focused on the purity of the saponins, since crude saponin preparations may need to be used in considerably higher doses. As a guideline, Dalsgaard *et al.* (1990) gave the following dose recommendations for Quil A (a semipurified and characterized preparation of adjuvant active saponins): 10 μg for use in mice, 50 μg in guinea-pigs, 200 μg in rabbits, 500 μg in pigs and 1000 μg in cattle. The amount of antigen can vary from microgram up to milligram quantities, depending on its intrinsic immunogenicity, although a range of 10–100 μg is typical for most vaccines.

Iscom-matrix

The Iscomatrix (Iscotec AB), or iscom-matrix (AdVet AB), is a particulate complex with identical composition, shape and appearance as the iscom (see below), except that it lacks inserted antigens (Lövgren and Morein, 1988). Since the iscom-matrix contains adjuvant-active Quil A, it can be used as an adjuvant simply mixed with antigens. A major advantage of using the iscom-matrix, compared with free saponin, is that the haemolytic activity of saponins in iscom-matrix is absent or drastically reduced (Kersten *et al.*, 1991; Rönnberg *et al.*, 1995). The saponins are bound to cholesterol within in the complex, and therefore do not bind to tissue cholesterol at the site of injection and thereby cause local reactions (Rönnberg *et al.*, 1995; B. Sundquist, personal communication). However, to date only limited information is available concerning the mechanism of adjuvant activity of iscom-matrix, especially in comparison to iscoms (Lövgren-Bengtsson and Sjölander, 1996; Cox *et al.*, 1997).

Type of immune modulation

The subclass distribution of antigen-specific serum antibodies in mice immunized with antigen mixed with iscom-matrix roughly parallel that in

mice immunized with iscoms. Likewise, antigen-specific spleen cells from mice immunized with iscoms or flu-Ag adjuvanted with iscom-matrix produce high levels of IL-2 and IFN-γ after restimulation (Lövgren-Bengtsson and Sjölander, 1996). Another feature of iscoms, the activation of cytotoxic T lymphocytes (CTLs), is reported after immunization with antigen adjuvanted with iscom-matrix (Cox *et al.*, 1997). However, these CTL responses were substantially weaker (about three-fold) than those induced by iscoms.

Iscom-matrix can lack the superior antigen-presenting ability of iscoms, because an antigen mixed with iscom-matrix may not be physically associated with the iscom-matrix. Like saponins in general, iscom-matrix is a potent and useful adjuvant for particulate or strong antigens (Jones *et al.*, 1995; Snodgrass *et al.*, 1995), but inferior with poor antigens (Lövgren and Sundquist, 1994). Iscoms are potent inducers of antibody responses in serum as well as in local and distal mucosal surfaces after intranasal administration. In contrast, intranasal administration of iscom-matrix mixed with antigen induce high titres of antibodies in serum, moderate titres in lung extracts, but low titres of antibodies in remote mucosal organs (gut and vagina) (B. Morein, K. Hu, J. Ekström and K. Lövgren-Bengtsson, unpublished).

Dosing

The dose recommendations for *Quillaja* saponins (see above) are likely to be valid also for iscom-matrix.

Lipid A

The outer cell membrane of Gram-negative bacteria comprises an amphipathic molecule known as lipopolysaccaride (LPS) or endotoxin. LPS is a potent adjuvant (Johnsson *et al.*, 1956), but also induces toxic reactions such as fever and lethal shock. It has been shown (Takada and Kotani, 1989) that most of the biological activities of LPS are exerted by lipid A, a structural component of LPS. Variants of lipid A can be obtained from a variety of bacterial species, varying in their degree of toxicity and immunomodulating activity (Alving, 1993). Lipid A is a disaccharide of glucosamine with two phosphate groups and five or six fatty acid chains of variable length, usually C_{12} to C_{16} (Rietschel *et al.*, 1985). Attempts to separate the toxic properties from the immunomodulating activities have resulted in molecules with substantially reduced toxicity, the best known being monophosphoryl lipid A (MPL). Other detoxified derivatives of lipid A have been obtained by succinylation and phthalyation (Schenk *et al.*, 1969; Chedid *et al.*, 1975).

Type of immune modulation

Lipid A is a potent immunomodulator that can be formulated in an aqueous mixture with an antigen, but due to its amphipathic properties it is often contained in liposomes or oil emulsions.

Macrophages stimulated by lipid A increase their phagocytic capacity facilitating uptake, processing and presentation of antigen (Unanue and Allen, 1987) and release of IL-1, IL-6, IL-8, granulocyte–macrophage colony stimulating factor (GM-CSF) and tumour necrosis factor (TNF) (Arend and Massoni, 1986; Henricson *et al.*, 1990; Nowotny, 1990; Astiz *et al.*, 1995). In addition, lymphocytes produce IFN-γ and IL-2 (Carozzi *et al.*, 1989; Odean *et al.*, 1990).

Dosing

In mice, the dose of lipid A derivatives may vary from about 10 to 300 μg given in saline, w/o emulsion, o/w emulsion, in liposomes alone or in liposomes mixed with other immunomodulators. Rats, guinea-pigs and rabbits are given about 100–300 μg.

MDP Derivatives

Muramyl dipeptide (MDP) is the minimum adjuvant-active component obtained from the cell wall of certain Gram-positive bacteria, particularly mycobacteria. MDP has diverse biological activities, from modulation of acquired and innate immune responses to modulatory effects on the central nervous system (Takada and Kotani, 1995). It is a strong but reactive immunomodulator in w/o emulsions. In order to reduce the side-effects and to improve the physical characteristics of the molecule, a large number of MDP derivatives, including lipophilic derivatives, has been prepared by chemical synthesis (for references, see Takada and Kotani (1995)). 6-*O*-Stearoyl-MDP (L18-MDP) (Azuma, 1992), 6-*O*-(tetradecylhexadecanoyl)-MDP (B30-MDP) (Azuma, 1992) and murabutide (*N*-acetylmuramyl-L-alanyl-D-glutaminyl-n-butyl ester) (Chedid *et al.*, 1982) are MDP derivatives reported to exert powerful adjuvant activity in experimental vaccines (Takada and Kotani, 1995).

MTP-PE (muramyl tripeptide–phosphatidylethanolamine, Ciba-Geigy Ltd, Chiron Corp.) is a lipophilic derivative (Braun *et al.*, 1995), which in liposomes is superior to its aqueous form in terms of both immunostimulation and toxicity (Schumann *et al.*, 1989).

◆◆◆◆◆◆ ADJUVANT FORMULATIONS: COMBINATIONS

Since different antigens require different adjuvant activities from an adjuvant, mixed formulations (or combinations) have been designed to combine several areas of adjuvant activity and to increase adjuvant versatility. Addition of immunomodulators such as lipid A or MDP derivatives, saponins or γ-inulin, along with the proper presentation of antigens afforded by co-polymers, o/w emulsions, $Al(OH)_3$, liposomes or the iscom structure, further increase the adjuvant activity. Although physic-

ally different, the adjuvant formulations described below all function by providing increased antigen presentation and targeting, together with immunomodulation.

Syntex Adjuvant Formulation

Syntex adjuvant formulation (SAF) is a typical adjuvant formulation designed to improve antigen presentation and antigen targeting, and has a specific immune modulation in the direction of Th1-type responses. The immune modulation is achieved by a MDP derivative, threonyl-MDP (Thr-MDP). *In vitro*, Thr-MDP stimulates IL-1 synthesis by guinea-pig macrophages and IL-1 and IL-6 synthesis by human peripheral mononuclear cells (Byars and Allison, 1995).

The antigen is mixed with Thr-MDP and the mixture of antigen and immunomodulator is mixed with a preformed o/w emulsion consisting of squalane, Pluronic L121 (a non-ionic block polymer) and Tween 80 emulsified in phosphate buffered saline (PBS) (Byars and Allison, 1995). The antigen is mixed with a preformed emulsion to avoid antigen denaturation caused by the shearing force of the emulsification process and to permit formulation by simple mixing.

Type of immune modulation

The SAF emulsion is described as microdroplets of squalane with Pluronic L121 on their surface. It is proposed that L121 allows the antigen to coat the droplets and bind complement components present in tissue fluids. Complement is activated via the alternative pathway. The antigen-coated microdroplets are carried to the surface of antigen-presenting cells, the antigen is endocytosed and processed, and peptides are exposed on the cell surface together with MHC class II molecules.

SAF formulations enhance both antibody production and cell-mediated immune responses. It enhances immune responses also in low responders such as young and old mice (Byars *et al.*, 1990, 1991); generally, in mice, the IgG2a isotype is particularly enhanced (Kenney *et al.*, 1989; Byars *et al.*, 1990, 1991).

Dosing

The final SAF formulation consists of 5% squalane, 2.5% Pluronic L121 and 0.2% Tween 80 in PBS (Vogel and Powell, 1995).

The dose of Thr-MDP is adjusted to fit the antigen and the animal species used. As a guideline, Byars and Allison (1995) recommend the use of about 50 µg Thr-MDP for guinea-pigs and mice (subcutaneous administration), 250–500 µg for rabbits, cats and monkeys (intramuscular or subcutaneous administration) and 5–20 mg for horses and ponies (intramuscular administration). The antigen dose is dependent on the immuno-

genicity of the antigen and the animal species being immunized. There is usually no need for more than two doses of vaccine.

Ribi Adjuvant System

The Ribi adjuvant system (RAS) is a preformed o/w emulsion containing various combinations of MPL, TDM and CWS, which like SAF only requires mixing with aqueous antigen. Aqueous forms of MPL generally stimulate antibody responses, and TDM and CWS tend to enhance cell-mediated responses. Consequently, a combination of MPL and TDM or CWS is recommended to obtain a mixed response (Rudbach *et al.*, 1995).

Dosing

The final emulsion contains 2% squalene, 0.2% Tween 80, and 0.25 mg ml^{-1} of each of MPL, CWS or TDM. For mice, 50 μg of each is recommended. The corresponding dose for rabbits and goats is 250 μg. The concentration of antigen should be in the range 50–250 μg ml^{-1}; weak antigens can be used at concentrations up to 1.0 mg ml^{-1}.

Iscom Adjuvant

The complex

Unlike the classical adjuvant formulations such as Freund's adjuvants and aluminium salts, which are blended with an antigen to formulate an emulsion or the antigen is adsorbed onto a three-dimensional gel, the iscom (immune stimulating complex, Iscotec AB) is a complex consisting of lipid, saponin and antigen. The complex will form only if the right constituents are allowed to interact in the correct stoichiometry.

The iscom complex is typically a 40-nm cage-like structure combining a multimeric presentation of antigen with a built in saponin adjuvant (Morein *et al.*, 1984), e.g. Quil A (Dalsgaard, 1978). The physical three-dimensional structure of the iscom is built up from 10- to 12-nm subunits formed by the interaction of Quil A with cholesterol (Özel *et al.*, 1989; Kersten *et al.*, 1991). By the addition of phospholipids, hydrophobic and amphipathic antigens are incorporated into iscoms by hydrophobic interactions during the assembly of the subunits (Lövgren and Morein, 1988).

In practice, iscoms are constructed by mixing antigens and saponin with detergent-solubilized cholesterol and phospholipid (Lövgren *et al.*, 1987). The detergent is removed by dialysis, ultrafiltration or ultracentrifugation, and iscoms form spontaneously during this process. To date, a large number of reports have been published describing different procedures for constructing iscom and the immune-enhancing properties of iscom preparations (Morein *et al.*, 1995; Rimmelzwaan and Osterhaus, 1995; Barr and Mitchell, 1996).

The experimental conditions for incorporation of different antigens and saponins into iscoms have mostly been empirical. Furthermore,

iscom preparations are mostly characterized by their protein content, largely neglecting the amount of incorporated saponin. As a consequence, little consideration has been given to the importance of the ratio between antigen and saponin so that the iscom has sufficient adjuvanticity but minimal toxicity (Lövgren-Bengtsson and Sjölander, 1996).

The iscom resulted from efforts to present antigens and adjuvant together in the same particle. Such an approach was expected to increase the immunogenicity of incorporated proteins and thereby decrease the required dose of both antigen and adjuvant. However, not all antigens spontaneously incorporate into iscoms using standard procedures, even if they are hydrophobic, and many antigens are too hydrophilic to incorporate into iscoms.

Basic constituents and prerequisites for iscom formation

Quillaja *saponin*

The basis for iscom formation is the interaction of *Quillaja* saponins with cholesterol. The action of saponins on biological membranes and their specific affinity for cholesterol has been known for many years (Dourmashkin *et al.*, 1962; Lucy and Glauert, 1964), but its practical application to the creation of the iscom is much more recent (Morein *et al.*, 1984). Not all saponins in a crude or semipurified extract form 'classical' 40-nm iscoms, even though some type of complex or aggregate is often formed when *Quillaja* saponins (Kersten *et al.*, 1991) or some other saponins (Bomford *et al.*, 1992) are incubated with cholesterol. There are preparations of *Quillaja* saponins available commercially that have been tested and selected for their iscom-forming ability: Quil-A (Superfos AS), Spikoside (AdVet) and Iscoprep 703 (Iscotec AB).

Lipids

Cholesterol is indispensable for iscom formation, since the unique affinity of *Quillaja* saponins for cholesterol is the cause of the complex formation. Many other sterols, such as cholesterol derivatives and plant sterols, also form complexes with saponins, with structures similar or identical to that of iscoms (K. Lövgren and B. Morein, unpublished), and saponins other than *Quillaja* saponins interact with cholesterol to form some sort of structure (Bomford *et al.*, 1992). However, so far, cholesterol and *Quillaja* saponins is the only suitable combination for the production of iscoms, at least for vaccine use.

Phospholipids are used as a supplementary lipid, even though other lipids can replace them. We once stated that iscom-matrix could be prepared from cholesterol and Quil A alone (Lövgren and Morein, 1988), although subsequently it was shown that the semi-purified MEGA-10 used in the preparation contained substantial amounts of decanoic acid which substituted for the phospholipids. The choice of phospholipid has always been an open question, and there have been few arguments to favour one over another. However, it has recently been noted that

phospholipids may have immunomodulatory effects in liposomes (Phillips *et al.*, 1996), and preliminary findings have also indicated that iscoms made with different phospholipids may differ in terms of immunogenicity and toxicity (L. Åkerblom and B. Morein, unpublished; L. Beezum, A. Coulter and J. Cox, unpublished). In terms of constructional aspects, phospholipids also have different characteristics. The choice of phospholipid is more restricted when the purified *Quillaja* saponin preparation Iscoprep 703, is used rather than semipurified *Quillaja* saponin preparations such as Quil-A (Superfos) or Spikoside (AdVet) (K. Lövgren-Bengtsson and L. Sandberg, unpublished).

Iscom formation

The basic instructions for making iscoms have been to take 1 mg each of detergent-solubilized cholesterol and phospholipid per milligram of antigen and to mix this with 5 mg saponin before extensive dialysis against, for example, PBS (Lövgren *et al.*, 1990).

The first method described for iscom formation was based on ultracentrifugation (Morein *et al.*, 1984). Detergent-solubilized membrane derived antigens were placed on the top of a 20–60% w/w sucrose gradient containing 0.05% saponin. Between the gradient and the antigen was a thin layer of 10% sucrose containing 0.1% Triton X100. This detergent-containing zone retains solubilized membrane lipids, but not those lipids associated with the membrane-anchoring parts of the antigen. Later, when the antigens carrying lipids enter the saponin-containing zone, iscoms are formed. Both the centrifugation method and the methods based on dialysis/ultrafiltration have advantages and disadvantages. The method of choice should be selected to suit the antigen and the desired composition of the iscom. Since the centrifugation method relies on the presence of lipids already associated with the antigen, this method is most suited for membrane antigens (native or recombinants) extracted from the membrane with a suitable detergent. Since the antigen is removed from the detergent-containing sample zone during the centrifugation, there are no other demands on the detergent than to solubilize the antigen gently without replacing the lipids and without denaturation of conformational epitopes stabilized by hydrophobic interactions (Merza *et al.*, 1989). Iscoms prepared using the centrifugation method will contain a minimum of lipid, and therefore also low amounts of saponin. Iscoms made from measles virus glycoproteins using the centrifugation method contained only 0.2 μg saponin per microgram of antigen (Morein *et al.*, 1984).

Compared to the dialysis/ultrafiltration methods, iscoms produced by the centrifugation technique are probably less toxic.

If the antigens carry too few lipids there will not be enough lipid to construct complexes with saponin, and the preparation will contain iscoms, iscom fragments, protein micelles or protein aggregates.

Many antigens are not membrane proteins, and even membrane-derived antigens need purification that might remove too much membrane lipid before incorporation into iscoms. Other antigens do not bind

very well to lipids in the presence of detergent, and will not bring their lipids down to the saponin-containing zone during centrifugation. For these antigens, the dialysis/ultrafiltration methods are most useful. As mentioned above, the antigens are mixed and incubated with a suitable amount of lipid and an adequate amount of saponin before the detergent is removed. During removal of the detergent, hydrophobic and amphipathic antigens associate with the iscom constituents and incorporate into the iscom particles. Even if the incorporation of the antigen fails, the iscom backbone consisting of saponin and lipids, the so called iscom-matrix, will form.

Antigen handling

The composition of an iscom should be chosen to tolerate the incorporation of an antigen into the structure without weakening the physical stability of the complex. In practice this has not been much of a problem, and most antigens, large or small, incorporate without disruption of the complex.

The handling of the antigen before incorporation is important. Since the antigens most suited for iscom formulation are hydrophobic or amphipathic, these must be purified in the presence of a detergent in such a way that they will not form protein micelles or aggregates. This is because pure protein micelles or aggregates are very difficult to dissociate, so that their hydrophobic part is accessible for hydrophobic interactions. There are many examples where antigens which theoretically should incorporate well in fact do not as a result of such aggregates.

Amphipathic/hydrophobic antigens

The iscom technology was originally developed for amphipathic/hydrophobic antigens, which generally incorporate nicely into iscoms. For many of these antigens, the choice of methodology is not important.

Some hydrophobic antigens are poorly soluble, even in detergents. Such antigens can be solubilized in, for example, dimethyl sulfoxide (DMSO), urea, ethylene glycol or guanidine hydrochloride (GuaHCl), and then lipid in detergent stock solution and saponin is added, as for other antigens. Initial dialysis/ultrafiltration is performed against a solution containing a lower concentration of solubilizer (DMSO, urea, ethylene glycol or GuaHCl), followed by a physiological buffer (e.g. PBS).

Hydrophilic antigens

Hydrophilic antigens do not incorporate into iscoms. To make iscoms containing hydrophilic antigen, hydrophobic regions must be introduced by, for example, binding lipids to them (Mowat *et al.*, 1991; Browning *et al.*, 1992; Reid, 1992; Scheepers and Becht, 1994) or exposed by, for example, low pH treatment. At first glance, one may think that such a treatment will irreversibly denature the antigens, and for some antigens this is true.

In fact, low pH treatment is often less denaturing than chemical modification (K. Lövgren-Bengtsson, B. Morein, J. Ekström, L. Akerblom, M. Villacrés-Eriksson, unpublished).

Low pH method (Morein *et al.*, 1990; Pyle *et al.*, 1989; Sjölander *et al.*, 1996b). The protein is mixed with lipid and the pH lowered generally to between 2.5 and 5, depending on the nature of the antigen. After a short incubation (about 1 h) at room temperature, the saponin is added and the mixture is incubated for 1–2 h before dialysis/filtration. The first volumes of buffer should have the same low pH as the initial mixture; the buffer is then changed to, for example, PBS. Using the detergent MEGA-10, a white precipitate may form at low pH. The precipitate consists of MEGA-10 and possibly some lipid, and will dissolve as soon as the pH is brought back to neutral.

Conjugation of antigens to preformed iscom-matrix. Antigen can be covalently linked to preformed iscom-matrix as an alternative to incorporation. The iscom-matrix used for conjugation of antigens is the same as that used as adjuvant (see above), with the exception that it contains a phospholipid with a functional polar head group suitable for conjugation (e.g. phosphatidyl ethanolamine) (Lundén *et al.*, 1997; Sjölander *et al.*, 1996b). The same chemistry as that described below for lipid modifications is applicable.

Lipid modification of antigens. There is a variety of protein modification and cross-linking reagents (e.g. Pierce) and activated lipids (e.g. Nothern Lipids) commercially available. The vast majority of these reagents rely on the presence of amino groups ($-NH_2$) that can be used for binding. Some reagents react with sulfhydryl groups (–SH) and a few methods are available for directed reaction involving carboxyl groups (–COOH) and carbohydrates.

- *Carbodiimide chemistry.* Conjugation of aliphatic carboxylic acids to amino groups in the antigen, or conjugation of amino acid carboxyl groups to amine containing lipid tails has been described by Sjölander *et al.* (1997b).
- *Active esters*, e.g. the conjugation of succinimide esters of aliphatic carboxylic acids to amino groups in the antigen (Mowat *et al.*, 1991; Browning *et al.*, 1992; Reid, 1992).
- *Sulfhydryl (–SH) reactive chemistry.* As with the carbodiimide reaction, sulfhydryl reactive groups can be used to attach amino groups to the antigen or to an amino group in the lipid tail. Sulfhydryl groups within the antigen can sometimes be made available for conjugation by dithiothreitol (DTT) reduction of intermolecular disulfide bonds (cys–S–S–cys). If reducible disulfide bonds are not present or are sterically hidden, sulfhydryl groups can be introduced by amino group modification or by modification of carbohydrates using commercially available reagents (Larsson *et al.*, 1993; Lövgren and Larsson, 1994; Sjölander *et al.*, 1996b).

Peptides/haptens

Synthetic oligopeptides and other low-molecular-weight haptens that require a carrier protein, i.e. B-cell epitopes, can be covalently linked to

preformed carrier iscoms using the techniques for covalent conjugation described above.

Oligopeptides or small proteins consisting of both T- and B-cell epitopes can, if they are amphipathic or hydrophobic, be incorporated using the same general methods described above or can be conjugated to a lipid prior to incorporation into iscoms (Pedersen *et al.*, 1992; Weijer *et al.*, 1993). Hydrophilic peptides can be conjugated to preformed iscoms containing carrier proteins (Sjölander *et al.*, 1991, 1993; Larsson *et al.*, 1993; Lövgren and Larsson, 1994).

Characterization of the final product: composition of iscoms

Whichever method is used for incorporating antigens into iscoms, the goal is the same – a highly immunogenic particle of low toxicity, containing an exposed antigen with antigenicity of the native molecule. The composition of the 'ideal' iscom is, of course, dependent on several factors, such as antigen immunogenicity, grade and purity of *Quillaja* saponin, the species to be immunized and the antigen dose to be used for immunization. Even with a strong antigen such as influenza virus membrane glycoproteins, an increased ratio (μg/μg) of *Quillaja* saponin to protein can increase the immunogenicity in such a way that a 10-μg dose of antigen in iscoms with a low proportion of Quil A (1 μg Quil A per μg antigen) is as immunogenic as a 1-μg dose of antigen in iscoms with a high proportion of Quil A (5 μg Quil A per mg antigen) (Lövgren-Bengtsson and Sjölander, 1996).

Problems

With proteins that spontaneously incorporate into iscoms there are generally no problems encountered during iscom preparation. The antigens incorporate quantitatively and it is easy to predict the composition of the iscoms produced. Most of the problems concerning iscoms arise when antigens that incorporate poorly (or not at all) are used and the resulting preparation is tested in animals without a proper analysis of the final product. There are several examples in the literature of poor or toxic iscoms resulting from problems with insufficient antigen incorporation.

In the study by Kersten *et al.* (1988) in which a strongly hydrophobic bacterial antigen was used, the iscoms were toxic at a dose of 2.5 μg antigen with a Quil A/protein ratio (w/w) of 20:1. In another study (Steinecker *et al.*, 1995) gp120 iscoms were not at all immunogenic, according to the authors, probably due to the fact that gp120 was not incorporated into the iscoms. However, by analysing the final iscoms to establish the antigen/Quil A ratio, suboptimal iscom preparations can be safety tested in laboratory animals. Special attention must be given to preparations for use in mice. Mice in general are sensitive to saponins, but there is a large strain variation. Also, the mode of administration to mice plays a substantial role. Intravenous and intraperitoneal administration should preferably be avoided. If necessary, the dose of iscoms should be reduced

to about 10% of that suitable for use subcutaneously or intramuscularly, i.e., 0.1–0.5 mg (antigen) or a dose of iscoms containing < 1 μg Quil A. The increased toxicity of intravenous and intraperitoneal administration is most probably related to a highly efficient and rapid uptake of the iscoms. In a dose–response study of iscoms administered intraperitoneally and subcutaneously, about a 10-fold lower dose was required intraperitoneally to generate the same magnitude of response as that of iscoms given subcutaneously (K. Lövgren-Bengtsson, unpublished).

Type of immune modulation

Iscoms enhance immune responses in various ways, including increased MHC class II expression on antigen-presenting cells (Bergström-Mollauglo *et al.*, 1992; Watson *et al.*, 1992), induction of IL-1 production (Villacrés-Eriksson *et al.*, 1993; Valensi *et al.*, 1994), activation of helper (Fossum *et al.*, 1990; Villacrés-Eriksson *et al.*, 1992) and cytotoxic T cells (Jones *et al.*, 1988; Takahashi *et al.*, 1990), and generation of potent long-lasting antibody responses (Lövgren, 1988; Sundquist *et al.*, 1988; Hannant *et al.*, 1993; Mumford *et al.*, 1994a,b), involving all subclasses and isotypes (Lövgren, 1988; Villacrés-Eriksson *et al.*, 1993). In the mouse a typical antibody response consists of equal proportions of IgG1 and IgG2, a medium proportion of IgG2b and minor but substantial amounts of IgG3.

The cellular response includes $CD8^+$ class I dependent CTLs and proliferating lymphocytes secreting IL-2 and high amounts of IFN-γ (Morein *et al.*, 1995) and IL-12 (Villacrés-Eriksson *et al.*, 1997). Antigen-presenting cells such as macrophages and dendritic cells produce IL-1 and IL-6 after *in vitro* stimulation with iscoms (Villacrés-Eriksson, 1995).

Iscom-borne antigen is rapidly taken up from the site of injection and transported to draining lymph nodes where there is a potent but transient response of proliferating T cells producing IL-2 and IFN-γ at days 4–8 after immunization; this is then transferred to the spleen (Sjölander *et al.*, 1997a). Iscoms also induce good immune responses following intranasal and oral administration (Lövgren, 1988; Mowat *et al.*, 1991).

Dosing

In general, the same maximum dose limitations as those mentioned for free Quil A (see above) are valid also for iscoms, although the dose of Quil A in iscoms required for a potent immune response is by far lower. The dose of antigen required, especially with monomeric antigens, may be one-tenth of that required with other adjuvant formulations (Morein *et al.*, 1995).

◆◆◆◆◆ SUMMARY

To choose the optimum adjuvant for a particular antigen preparation is not easy and there are many factors to consider. Of major importance is whether the purpose of the immunization is to develop a vaccine or to raise antibodies for use in immunoassays or affinity chromatography. Antigen availability and choice of animal model are also often issues of primary concern. Secondly, we must consider the physical characteristics of the antigen: is it likely to be immunogenic or is it one of those small hydrophilic antigens that are usually poorly immunogenic?

If there is only a small quantity of a hydrophilic antigen available and the purpose is to raise antibodies for use in immunoassay, one of the most reliable adjuvants is FCA. Although efficient, FCA does have disagreeable side-effects, and for the well-being of laboratory animals the use of FCA is discouraged or forbidden in many countries. The modern commercially available alternatives that are equally simple to use are the oil emulsion type adjuvants such as TiterMax and Ribi adjuvants. If the antigen is not in short supply and there is time for some development work, the incorporation into iscoms will generate a highly immunogenic formulation. With particulate antigens there is a wider choice of adjuvants, and the choice and efforts can be focused on obtaining the desired immune response.

For vaccine development many factors must be considered, and it is beyond the scope of this chapter to give a rationale for the choice of adjuvant, although several of the adjuvants discussed are either in clinical trials in humans or already in use in commercially available animal vaccines.

The market for commercially available and experimental adjuvants is increasing. Adjuvants are becoming more and more efficient and versatile, although we will probably never see the day when one adjuvant can replace all the others, as antigen preparations are different and the characteristics of a protective immune response vary from disease to disease.

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PART III

Human System

1 Isolation and Propagation of Human Dendritic Cells
2 Isolation of T Cells and Establishment of T-cell Lines and Clones
3 Growth Transformation of Human T Cells
4 Generation and Characterization of Killer Cells
5 Measuring Human Cytokine Responses
6 Measuring Immune Responses *In Situ*: Immunohistology and *In Situ* Hybridization
7 Isolation, Characterization and Cultivation of Human Monocytes and Macrophages

1 Isolation and Propagation of Human Dendritic Cells

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Introduction
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INTRODUCTION

Dendritic cells (DCs) represent a trace population of cells, expressing high levels of major histocompatibility complex (MHC) class II antigens, with a characteristic dendritic shape. They are bone-marrow-derived professional antigen-presenting cells (APCs), which are required for initiation of immune responses. They comprise a system that occupies: (a) discrete areas of non-lymphoid tissues with the Langerhans cells of epithelium (skin, mucosa, lung) and the interstitial DCs of the heart, kidney and virtually all other organs; (b) the circulation with the afferent lymph veiled cells and the peripheral blood DCs; (c) the T-cell-rich areas within secondary lymphoid organs, where they are the interdigitating cells (IDCs); (d) the thymic medulla; and (e) the B-cell follicles of secondary lymphoid organs (Grouard *et al.*, 1996), where they are the germinal centre DCs (GCDCs) (for reviews see: Metlay *et al.*, 1989;

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Streilein and Grammer, 1989; Hart and McKenzie, 1990; Steinman, 1991; Knight and Stagg, 1993; Williams *et* al., 1994; Caux and Banchereau, 1996; Girolomoni and Ricciardi-Castagnoli, 1997). Although the relationship between the various DC populations is not yet fully understood, they are thought to represent different steps of maturation interconnected by defined pathways of circulation (Steinman, 1991). In the periphery, DCs such as Langerhans cells (LCs) capture antigens (Inaba *et al.*, 1986; Romani *et al.*, 1989; Streilein and Grammer, 1989; Puré *et al.*, 1990), and then migrate via the lymphatics or blood vessels and home to the T-cell-rich areas of secondary lymphoid organ (IDCs) (Macatonia *et al.*, 1987; Austyn *et al.*, 1988; Fossum, 1988; Kupiec-Weglinski *et al.*, 1988; Kripke *et al.*, 1990; Larsen *et al.*, 1990; De Smedt *et al.*, 1996; Kudo *et al.*, 1997). There, they present processed antigen to naive T cells and generate an antigen-specific primary T-cell response (Inaba *et al.*, 1990b; Sornasse *et al.*, 1992; Liu and MacPherson, 1993). This T-cell priming is then followed by the extrafollicular primary B-cell response (Inaba and Steinman, 1985; Sornasse *et al.*, 1992; Flamand *et al.*, 1994). During their migration, from periphery to draining lymph nodes, DCs undergo modulations of phenotype and functions, including loss of antigen processing and acquisition of accessory function (Schuler and Steinman, 1985; Romani *et al.*, 1989; Streilein and Grammer, 1989; Larsen *et al.*, 1990).

Defining the mechanisms of DC action is important, as it may ultimately permit an understanding of how T cells are primed. This knowledge should allow the manipulation of the immune responses at the early sensitization phase of immunity: down-regulation in case of autoimmunity or transplantation and enhancement in case of infectious diseases or cancer. In addition, understanding DC physiology has implications in the context of viral infections, insofar as DCs appear to be targets for viruses such as human immunodeficiency virus (HIV), human T-cell leukaemia/lymphoma virus type 1 (HTLV-1), measles virus, influenza virus and herpes simplex virus (HSV) and might be involved in virus spreading and general immune suppression (Knight *et al.*, 1991; Langhoff *et al.*, 1991; Cameron *et al.*, 1992, 1994; Ali *et al.*, 1993; Dezutter-Dambuyant and Schmitt, 1993; Bhardwaj *et al.*, 1994a; Pope *et al.*, 1994; Weissman *et al.*, 1995; Ghanekar *et al.*, 1996; Bhardwaj, 1997; Fugier-Vivier *et al.*, 1997; Grosjean *et al.*, 1997).

While the bone marrow origin of DCs was recognized 20 years ago, it is only recently that the conditions that direct their growth and differentiation have been deciphered. Granulocyte–macrophage colony-stimulating factor (GM–CSF) appears to act as a central factor for DC development, both in mice and in humans. As DCs are difficult to isolate from tissues, their *in vitro* generation represents an important step towards the understanding of DC physiology and their potential use in immunotherapy.

Herein we describe (a) the isolation of different DC populations from tissues; (b) the *in vitro* culture conditions that allow the generation of DCs from haematopoietic progenitors or from circulating blood monocytes; and (c) the assessment of the functional properties of these DCs.

◆◆◆◆◆◆ ISOLATION OF DENDRITIC CELLS

Isolation from Human Skin

Characteristic features

The stratified squamous epithelium (skin and mucosa) is the first line of defence of the organism against external agents, not only as a physical barrier between the body and the environment, but also as the site of initiation of immune reactions. Human epidermis is a heterogeneous epithelium composed of keratinocytes, melanocytes, Merkel cells and APCs: the Langerhans cells (LCs) (Stingl and Shevach, 1991). LCs represent 1–6% of the epidermal cells (ECs) (Romani *et al.*, 1991) and can be

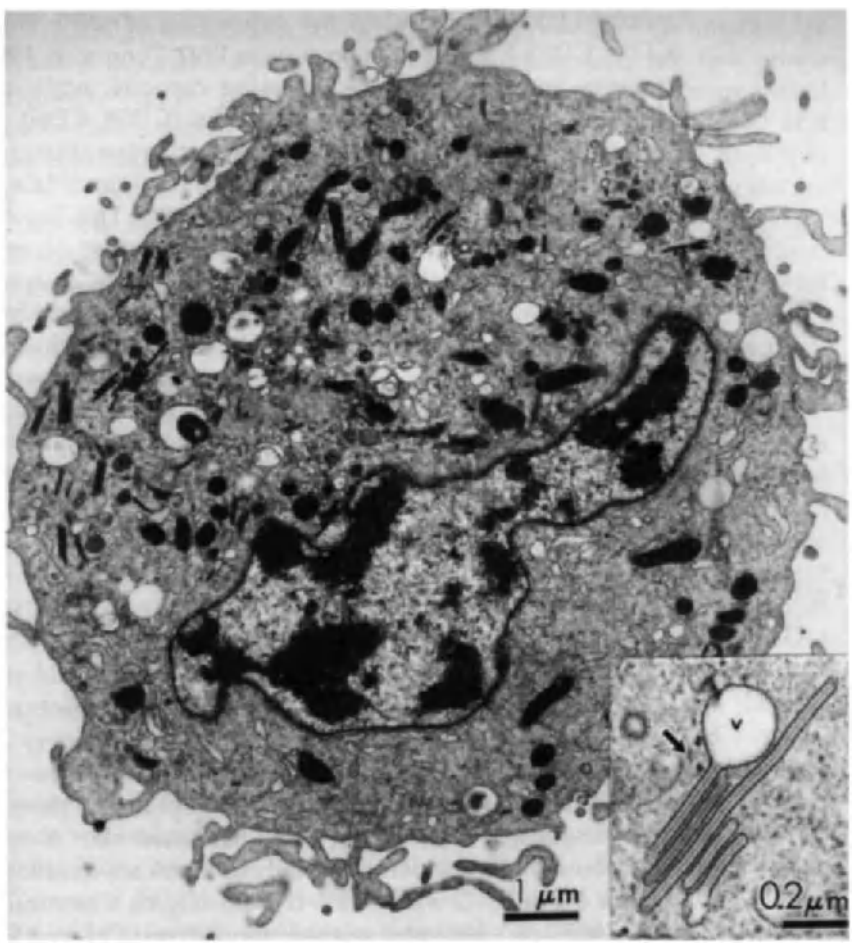


Figure 1. Electron microscopic view of epidermal Langerhans cells. Transmission electron micrograph of Langerhans cell freshly isolated from epidermal suspensions. Low-power view: a Langerhans cell with a lobulated nucleus, numerous dendrites and a clear cytoplasm with various organelles. High-power view: the Birbeck granules (arrows) appear as rod-shaped structures of variable length with a central, periodically striated lamella or as tennis-racket-like structures where part of the rod opens in a vesicle (v).

distinguished from the surrounding ECs by the expression of the MHC class II, CD1a, CD4, CD11b/CD18, CD11c/CD18, CD32 (FcγRII), CD45RO, CD50 (ICAM-3), CD58 (LFA-3) and CD86 (B7-2 antigen) antigens, and by the presence of rod-shaped cytoplasmic organelles, called Birbeck granules (Birbeck *et al.*, 1961; Schmitt *et al.*, 1990; Dezutter-Dambuyant, 1991; Dezutter-Dambuyant *et al.*, 1991; Romani *et al.*, 1991; Teunissen, 1992) (Fig. 1). Originating from the bone marrow, LCs colonize the epithelia where they function as sentinels of the immune system by acting as APCs, taking and processing antigens, and migrating to T-cell-dependent areas of regional lymph nodes where they sensitize T cells and induce an MHC-restricted antigen-specific primary immune response. Freshly isolated epidermal LCs are weak stimulators of naive T cells, but are effective processors of soluble protein antigens. After short-term culture LCs down-regulate the expression of some surface molecules (CD1a, CD32, E-cadherin) (Teunissen, 1992; Tang *et al.*, 1993), lose Birbeck granules and their antigen-processing capacity, while they upregulate MHC class I and II, accessory molecules (CD58, CD80, CD86) (Péguet-Navarro *et al.*, 1993) and acquire some activation/maturation molecules (CD25, CD83) (Zhou and Tedder, 1995). Cultured LCs become potent immunostimulatory cells (Romani *et al.*, 1989). This *in vitro* phenotypical and functional maturation process mimics what occurs *in vivo* when perturbation of the skin milieu causes migration of epidermal LCs via dermal lymphatic vessels to draining lymph nodes and maturation of the migrating LCs from effective APCs into potent immunostimulatory APCs. *In vivo* they are responsible for the induction of contact hypersensitivity to haptens (Kripke *et al.*, 1990; Enk and Katz, 1992) and take part in skin graft rejection and in presentation of bacterial, viral and tumour antigens to T cells (Dezutter-Dambuyant, 1991; Dezutter-Dambuyant *et al.*, 1991; Grabbe and Granstein, 1995; Moll *et al.*, 1995).

Technical aspect

Isolation of epidermal cells

Epidermal cell suspensions are obtained from fresh normal skin of patients undergoing reconstructive plastic surgery of breast or abdomen. A trypsinization technique allows the separation of epidermis from dermis and disrupts the epidermal sheets into a single-cell suspension (Ray *et al.*, 1989). Trimmed skin is split-cut with a keratotome set. The dermo-epidermal slices are treated for 18 h at 4°C or for 1 h at 37°C with 0.05% trypsin (Sigma Chemical Co, St Louis, MO) in Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (Seromed, Biochrom KG, Berlin). Such a low trypsin concentration is a good balance between tissue digestion and damage to surface molecules. The epidermis is then detached from the dermis with fine forceps. The epidermal sheets are pooled in medium 199 Hank's (Seromed, Biochrom KG, Berlin) supplemented with 20% fetal calf serum (FCS; Gibco BRL, Paisley, Scotland) (TC199/FCS) to stop trypsin action.

They are then vigorously and repeatedly blown in and out of a 5-ml pipette. After filtration through sterile gauze, the suspended cells are washed three times with TC199/FCS. Cells are counted and the viability is estimated by Trypan Blue exclusion. Viability after trypsinization must range from 80% to 95%.

Enrichment of epidermal dendritic cells or Langerhans cells

LC enrichment is achieved by successive density gradient centrifugation steps and depletion of basal keratinocytes. Total EC suspensions (4×10^6 cells ml^{-1}) in TC199/FCS are layered on the Lymphoprep (Nycomed Pharma SA, density 1.077) with an EC suspension/Lymphoprep ratio of 2.5 : 1, and centrifuged for 30 min at 400*g*. The cells from the interface are washed twice and resuspended in TC199/FCS. After this first centrifugation step, LC enrichment reaches 7–15% LCs. The cells are plated onto dishes coated with collagen type 1 (IWAKI Glass, Japon) at a concentration of 5×10^6 to 6×10^6 cells ml^{-1} and incubated for 20–30 min at 37°C in order to plate the basal keratinocytes. Non-adherent cells are collected and layered on the diluted Lymphoprep (Lymphoprep/distilled water ratio 3.4 : 1.6) and centrifuged for 20 min at 400*g*. The low-density fraction at the interface is collected and washed three times with 10% FCS-supplemented RPMI-1640 medium (Gibco BRL, Paisley, Scotland). This last enrichment procedure (depletion of basal keratinocytes and modified gradient sedimentation) leads to a suspension containing about 70–95% LCs.

Further enrichment may be performed with a positive selection procedure which involves binding a ligand (e.g. anti-CD1a monoclonal antibody (mAb)) to the cell surface, but this may interfere with functional experiments. EC suspensions are suspended in phosphate buffered saline (PBS) containing 10 mM ethylene diamine tetraacetic acid (EDTA) and 0.5% bovine serum albumin (BSA; Boehringer Mannheim GmbH, Germany) (PBS/EDTA/BSA) and incubated with anti-CD1a mAb (OKT6, FITC-conjugate, 1/5 dilution, Ortho Diagnostics) at a final concentration of 10^7 cells ml^{-1} for 15 min at 4°C. After washes, the cells are incubated with goat anti-mouse immunoglobulin G (IgG) coated microbeads (20 µl bead suspension per 10^6 cells; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). After 15 min at 4°C, ECs are washed once and isolation of CD1a-positive cells is performed using Minimacs separation columns (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). ECs are suspended in PBS/EDTA/BSA at a concentration of 2×10^6 ml^{-1}, layered on the top of the column fixed under the magnet. After washing with 2×500 µl, the column is removed from the separator and placed on a suitable tube; the retained cells are eluted with 2 ml PBS/EDTA/BSA buffer using the plunger. The cells are centrifuged and then resuspended in 10% FCS-supplemented RPMI-1640 medium. The isolated cells contained 95–99% LCs stained with a fluorescein isothiocyanate (FITC)-conjugated anti-CD1a MAb (see Fig. 1).

Isolation from Human Peripheral Blood

Characteristic features

In human blood, although DCs are the main APCs (Freudenthal and Steinman, 1990; Thomas *et al.*, 1993) they represent 0.3–0.8% of leukocytes. Mature DCs display a high motility, continually extending and retracting large lamellipodia, or 'veils', a property that is not shared by other cells in blood. They express the DC maturation marker CD83 and form rosettes with T cells *in vivo* (Weissman *et al.*, 1995; Zhou and Tedder, 1995). However, in conventional protocols of purification involving T-cell depletion, freshly isolated DCs are immature and appear as medium-sized lymphocytes that lack typical DC morphology and phenotype (Young and Steinman, 1988; O'Doherty *et al.*, 1993; Thomas *et al.*, 1993; Zhou and Tedder, 1995). Upon 2 days of culture, blood DCs mature into cells displaying typical DC phenotype, morphology and function (O'Doherty *et al.*, 1993). Two subsets of blood DCs have been identified on the basis of CD11c expression, $CD11c^-$ cells (also $CD45RA^+$, $CD45RO^-$, $CD13^-$) being dependent on exogenous factors for their maturation, while $CD11c^+$ cells (also $CD45RA^-$, $CD45RO^+$, $CD13^+$) are capable of spontaneous maturation (O'Doherty *et al.*, 1994). It is hypothesized that the $CD11c^-$ cells are bone marrow derived precursors to tissue DCs, such as epidermal LCs, while the $CD11c^+$ cells arise from tissue where they have been activated by antigen and are en route to the spleen or lymph nodes. These $CD11c^-$ and $CD11c^+$ subsets might correspond to, respectively, the $CD14^{low}$ $CD33^+$ and $CD14^{low}$ $CD33^{bright}$ subsets described independently (Thomas *et al.*, 1993).

Technical aspect

Isolation of blood mononuclear cells

Blood (450 ml) is collected over CPD (Sigma), and diluted 50% with PBS. The suspension is layered on Ficoll-Hypaque and, after 30 min centrifugation, the mononuclear cells are recovered and washed with PBS.

Depletion of T, B and NK cells and monocytes

Cells are resuspended at 2×10^7 cells ml^{-1} in PBS containing 2% human serum (HS) and 0.5 mM EDTA and stained with anti-CD3 (OKT3, ascites 1/1000), anti-CD14 (MOP9 ascite 1/1000), anti-CD16 (ION16, Immunotech, 2.5 µg ml^{-1}), anti-CD19 (4G7, ascite 1/1000), anti-CD56 (NKH1, Ortho, 1 µg ml^{-1}) and anti-glycophorin A (Immunotech, 2.5 µg ml^{-1}). After 20–30 min at 4°C under gentle shaking, cells are washed three times with PBS containing 2% FCS and 0.5 mM EDTA, and re-suspended at 5×10^7 cells ml^{-1}. Goat anti-mouse Ig beads (Dynabeads, Dynal, Oslo, Norway) are added to the cell suspension (5 beads per cell,

original bead density 4×10^8 ml^{-1}). After 20–30 min at 4°C under gentle shaking, cells are incubated over the magnet for 10 min. Supernatants containing unbound cells are harvested. Beads are washed twice under the magnet. Collected supernatants are mixed and, after centrifugation, cells are adjusted at 5×10^7 ml^{-1} and submitted to another round of bead depletion. The resulting suspension, which contains 20–60% of dendritic cells (mean ± SD = 35 ± 25) represents 2.5–7.5% of the original suspension (450 ml blood yields 4.5×10^8 to 10×10^8 mononuclear cells (MNC), which yields 8×10^6 to 30×10^6 enriched cells). At this stage cells can be stored at 4°C overnight in RPMI complete medium.

FACS sorting of peripheral blood DC subsets

The method is based on a previously published one (O'Doherty *et al.*, 1994). Cells are resuspended at 2×10^7 cells ml^{-1} in PBS/HS/EDTA and stained with biotinylated anti-HLA-DR (Becton Dickinson), phycoerythrin (PE) coupled anti-CD11c (Becton Dickinson) and a cocktail of FITC coupled mAbs: anti-CD3, -CD14, -CD15, -CD16, -CD20, -CD57 (Becton Dickinson) and -CD34 (Immunotech). After 20–30 min at 4°C under gentle shaking, cells are washed three times with PBS/FCS/EDTA, resuspended at 2×10^7 cells ml^{-1}, and incubated with Tricolor Streptavidin (Caltag) for 20–30 min at 4°C under gentle shaking. After three washes cells are resuspended at 1×10^6 to 2×10^6 cells ml^{-1} and filtered over a 7-µm membrane (Filcons, Dako, Denmark). Cells are separated according to lack of FITC staining and expression of high level of HLA-DR and CD11c into FITC$^-$HLA-DR$^+$CD11c$^+$ and FITC$^-$HLA-DR$^+$CD11c$^-$ fractions using a FACStarplus (Fig. 2A). The CD11c$^+$ DC subpopulation represents 35–60% of the DR$^+$ pool. After sorting, 0.5×10^6 to 1.5×10^6 of each DC subset is recovered from 450 ml blood.

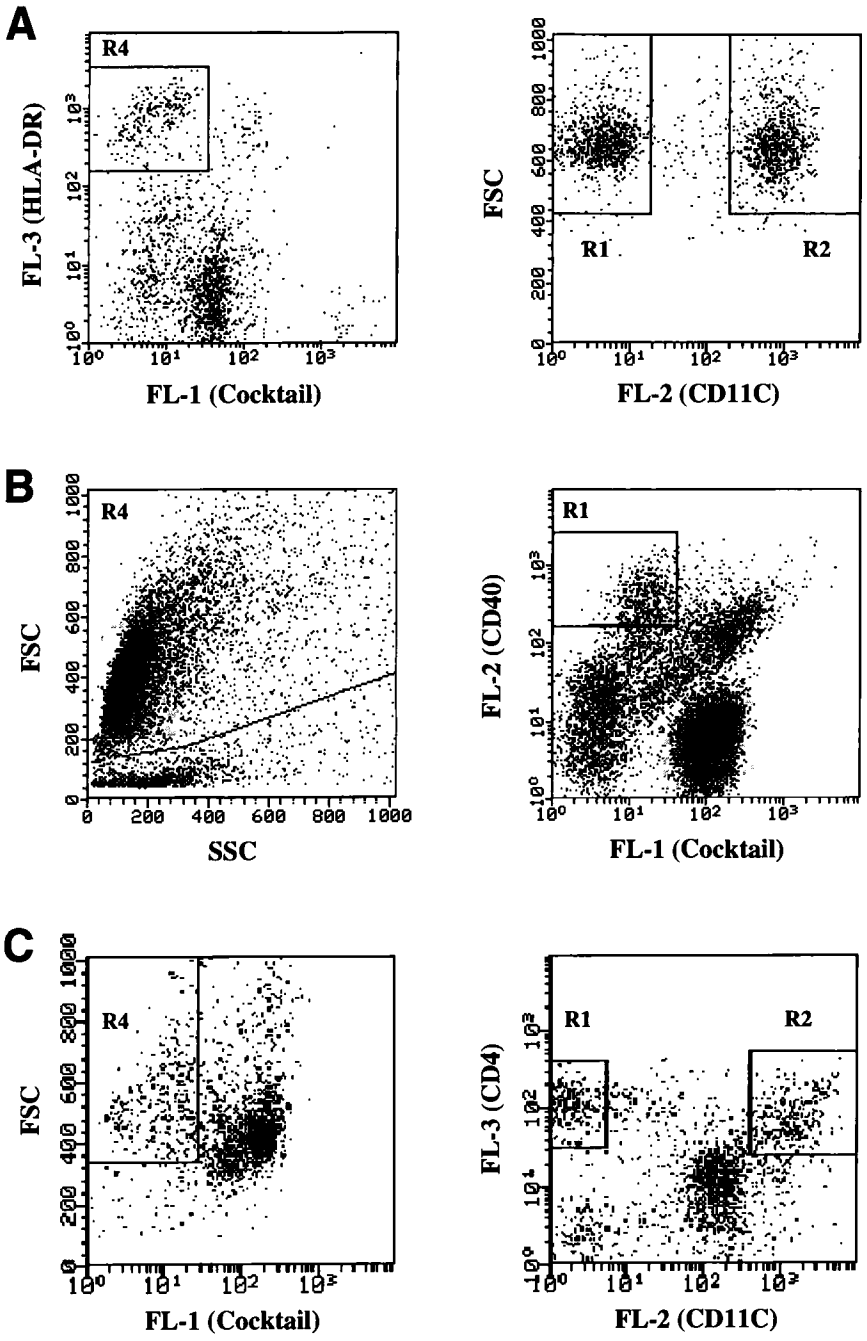
Isolation of DC Subsets and DC Precursors from Human Tonsils

In human tonsils, part of the mucosal associated lymphoid tissues, different subsets of DCs and DC precursors have been identified by immunohistology, including CD1a$^+$ Langerhans cells in mucosal epidermis, CD40$^+$CD80$^+$CD83$^+$CD86$^+$ IDCs, CD4$^+$CD3$^-$CD11c$^-$ plasmacytoid DC precursors within the T-cell rich areas and CD4$^+$CD3$^-$CD11c$^+$ DCs in GCDCs (Plate 7). In this section, the methods for isolating IDC, GCDC and CD4$^+$CD3$^-$CD11c$^-$ plasmacytoid DC precursors are described.

Interdigitating cells of T-cell area

Characteristic features

IDCs (Plate 7C) represent mature DCs, characterized by long interdigitating dendrites, expression of high levels of MHC class II, CD40, CD80,



CD83 and CD86, moderate levels of CD4 and very low levels of myeloid antigen CD13 and CD33 (Björck *et al.*, 1997). They may be derived from epidermal Langerhans cells, that migrate into the T-cell areas of draining lymphoid tissues after capture and processing of antigens (Steinman, 1991). These cells are thought to play a key role in priming antigen-specific naive T cells.

Isolation of IDC

Tonsils are cut into small pieces and digested for 12 min at 37°C with collagenase IV (1 mg ml^{-1}, Sigma) and deoxyribonuclease I (50 KU ml^{-1}, Sigma) in RPMI 1640. The cells, pooled from two rounds of tissue digestion, are centrifuged over 50% Percoll (Pharmacia) for 2 min at 400*g*. The resulting low-density cells are collected and subjected to magnetic bead depletion of T, B and NK cells and monocytes as described above. The resulting cells are labelled with a biotinylated antibody to CD40 (mAB89) and FITC-conjugated antibodies to CD19 (Immunotech), CD3, CD20, CD14 and CD57 (Becton-Dickinson). Cells are further incubated with Tricolor-labelled streptavidin (Caltag, San Francisco, CA). The CD40-only expressing cells are sorted as IDCs (Fig. 2B).

$CD4^+CD3^-CD11c^+$ GCDCs

Characteristic features

A population of large dendritic $CD4^+CD3^-$ cells was recently found within germinal centres (GCs) of human lymph nodes, tonsils and spleens (Grouard *et al.*, 1996) (Plate 7). Double colour immunohistochemistry reveals these $CD3^-CD4^+$ cells to be $CD1a^-$, $CD40^{low}B7^{low}$ and $DRC\text{-}1^-$ (Liu *et al.*, 1997), indicating that these cells are not Langerhans cells ($CD1a^+$), interdigitating cells (CD40 high, $B7.1/CD80^{high}$, $B7.2/CD86^{high}$) or FDCs ($DRC\text{-}1^+$, $KIM4^+$). These cells can be isolated from tonsillar mononuclear cells after magnetic bead depletion of T, B and NK cells and monocytes, followed by three-colour FACS sorting of $CD4^+CD11c^+lin^-$ cells (where lin^- is lineage). The isolated cells are characterized by a strong MHC class II expression and a morphology of dendritic cells (Plate 7d and Fig. 3). These cells display potent stimulatory activity on $CD4^+$ T cells. These dendritic cells probably stimulate the GC T cells that are required for the generation of memory B cells, and may contribute to the unique feature of HIV pathology within GCs. The presence of similar $CD4^+CD11c^+$ DCs in blood suggests that GCDCs were derived from precursors in blood.

Figure 2. FACS sorting of PB-DC, IDC, GCDC and $CD11c^-$ precursors. Mononuclear cells from blood or tonsils are subjected to magnetic bead depletion of T, B and NK cells and monocytes (see text). After staining, cells were sorted in different populations using a FACStarplus. (A) FACS sorting of $CD11c^+$ and $CD11c^-$ DC subsets from adult blood. Cells are separated according to lack of FITC staining (CD3, CD14, CD15, CD16, CD20, CD34, CD57) and expression of high level of HLA-DR (Tricolor) and CD11c (PE) into $FITC^-HLA\text{-}DR^+CD11^+$ and $FITC^-HLA\text{-}DR^+CD11c^-$ fractions. (B) FACS sorting of IDC from tonsils. Cells are separated according to lack of FITC staining (CD3, CD14, CD19, CD20, CD57) and expression of a high level of CD40 (Tricolor). (C) FACS sorting of $CD11c^+$ (GCDC) and $CD11c^-$ DC subsets from tonsils. Cells are separated according to lack of FITC staining (CD1a, CD3, CD7, CD14, CD16, CD20, CD34, CD57) and expression of CD4 (Tricolor) and CD11c (PE) into $FITC^-CD4^+CD11^+$ and $FITC^-CD4^+CD11c^-$ fractions.

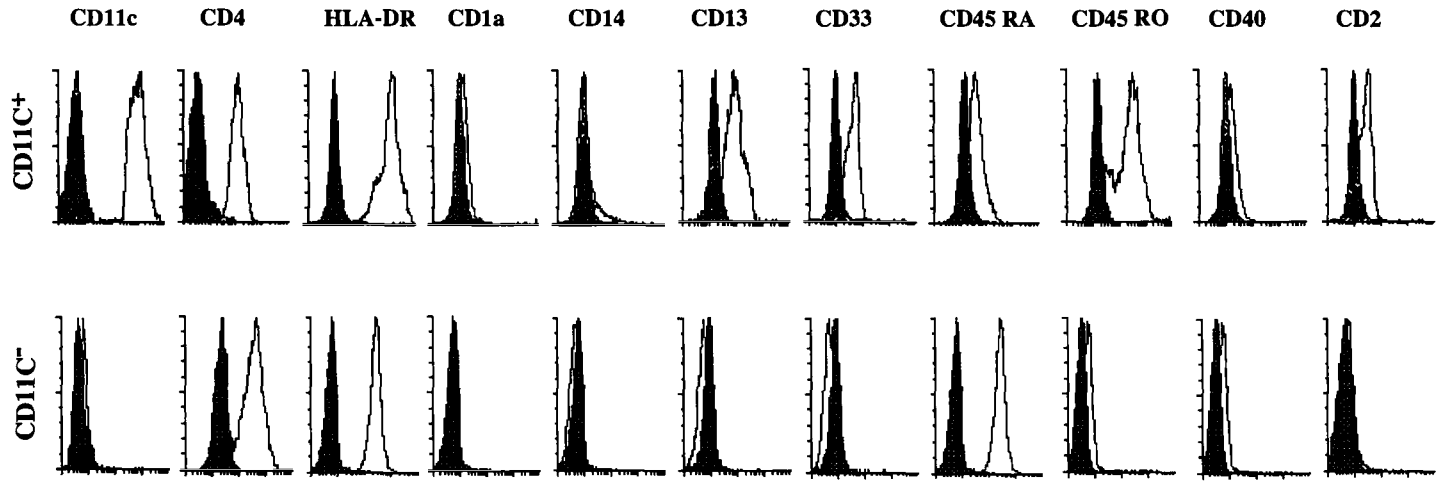


Figure 3. Phenotypic comparison of $CD11c^+$ and $CD11c^-$ DC subsets from tonsils. After FACS sorting, freshly isolated $CD11c^+$ and $CD11c^-$ populations were stained using FITC-coupled mAbs.

Technical aspect

Isolation of GCDC

Tonsillar cells are depleted of T, B and NK cells and monocytes, and stained with mouse anti-CD4-PE-Cy5 (Immunotech), anti-CD11c-PE (Becton Dickinson) and a cocktail of FITC-labelled mAbs, including anti-CD34 (Immunotech), -CD3, -CD20, -CD57, -CD7, -CD14 and -CD16 (Becton Dickinson) and -CD1a (Ortho). Then $CD4^+CD11c^-lin^-$ cells are isolated by cell sorting (Fig. 2C).

$CD4^+CD3^-CD11c^-$ plasmacytoid DC precursors

Characteristic features

A subset of $CD4^+CD11c^-CD3^-$ blood cells was recently shown to develop into dendritic cells when cultured with monocyte-conditioned medium (O'Doherty *et al.*, 1994). These cells correspond to the so-called 'plasmacytoid T cells', an obscure cell type that has long been observed by pathologists within secondary lymphoid tissues (Plate 7B,E). They express CD45RA, but no markers specific for known lymphoid- or myeloid-derived cell types (Fig. 3). They undergo rapid apoptosis in culture, unless rescued by IL-3. Further addition of CD40 ligand results in their differentiation into dendritic cells that express low levels of myeloid antigens CD13 and CD33. These cells may represent the precursors of lymphoid DCs in humans (Grouard *et al.*, 1997).

Technical aspect

Isolation of $CD4^+CD3^-CD11c^-$ plasmacytoid DC precursors

This population of cells can be isolated at the same time as the $CD4^+CD3^-CD11c^+$ cells (see above) (Fig. 2C).

◆◆◆◆◆◆ PROPAGATION OF DENDRITIC CELLS *IN VITRO*

Considerable progress has recently been made in generating DCs from mouse and human precursors. Consequently, culture systems are now available for the *in vitro* generation of high numbers of DCs.

Isolation from Human Peripheral Blood Monocytes

Characteristic features

In the mid-1980s, Knight *et al.* (1986) described monocytes that have acquired a veiled and dendritic appearance after separation. More recently, monocytes were induced to express CD1a after treatment with GM-CSF and IL-4 (Porcelli *et al.*, 1992) or GM-CSF alone (Kasinrerk *et al.*,

1993). This phenomenon is observed only with monocytes and only with GM-CSF (Kasinrerk *et al.*, 1993). It is now well established that monocytes can be induced, without any proliferation, to differentiate into $CD1a^+$ DCs, upon culture with GM-CSF and IL-4 (Romani *et al.*, 1994; Sallusto and Lanzavecchia, 1994; Pickl *et al.*, 1996; Zhou and Tedder, 1996) or IL-13 (Piemonti *et al.*, 1995). The monocyte-derived DCs display a phenotype of immature DCs, including low CD80, CD86, CD58 expression, expression of MHC class II within intracytoplasmic compartments, and expression of monocyte markers (CD11b, CD36, CD68, cfms). The cells display an efficient antigen uptake by macropinocytosis or by receptor-mediated endocytosis through the mannose receptor and a weak capacity to prime naive T cells. These DCs can undergo maturation when stimulated by signals such as lipopolysaccharide (LPS), tumour necrosis factor α (TNFα), interleukin 1 (IL-1) (signals also inducing DC migration) or by T-cell signals such as CD40L (Sallusto and Lanzavecchia, 1994; Sallusto *et al.*, 1995). The DCs then display a mature phenotype including a typical morphology with extended dendrites, a loss of monocyte markers, a loss of antigen uptake, an upregulation of accessory molecules (CD80, CD86, CD58), a translocation of MHC class II onto the cell surface, and a strong capacity to prime naive T cells.

Technical aspects

Purification of monocytes

Following a Ficoll-Hypaque (see p. 510), monocytes are enriched through 50% Percoll. The interface population usually contains 50–80% $CD14^+$ monocytes. Monocytes are purified further by depletion of contaminating T, B and NK cells using mAbs anti-CD3, -CD16, -CD19, -CD56 and -glycophorin A, and goat anti-mouse Ig beads (see p. 510). After two rounds of bead depletion the resulting suspension contains 95–99% monocytes as judged by anti-CD14 staining. A 450-ml aliquot of blood yields 30×10^6 to 150×10^6 monocytes.

Enrichment of monocytes by adherence is avoided in order not to interfere with subsequent differentiation into DCs.

Generation of dendritic cells from monocytes

Cultures are established in endotoxin-free medium consisting of RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS) (Flow Laboratories, Irvine, UK), 10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES), 2 mM L-glutamine, 5×10^{-5} M β_2-mercaptoethanol, penicillin (100 U ml^{-1}) and streptomycin (100 µg ml^{-1}) (referred to as 'complete medium'). Purified monocytes are seeded at 2.5×10^5 cells ml^{-1} (25 ml in 75 cm^2 or 50 ml in 150 cm^2 Falcon flasks) in RPMI complete medium

(10% FCS) containing 100 ng ml^{-1} GM-CSF (specific activity 2×10^6 U mg^{-1}; Schering-Plough Research Institute, Kenilworth, NJ) and 50 U ml^{-1} IL-4 (specific activity 10^7 U mg^{-1}; Schering-Plough Research Institute, Kenilworth, NJ). Cells are usually diluted by half at day 4–5 and recovered at day 8–10. At the end of the culture the number of DCs represents 40–60% of the number of seeded monocytes (Sallusto and Lanzavecchia, 1994; unpublished results).

Isolation from Human CD34$^+$ Haematopoietic Progenitor Cells

Characteristic features

TNFα in association with GM-CSF or IL-3 induces development of dendritic cells from CD34$^+$ cells

Tumor necrosis factors (α or β) strongly potentiate the proliferation induced by either IL-3 or GM-CSF of CD34$^+$ haematopoietic progenitor cells (HPCs) isolated from cord blood or bone marrow mononuclear cells (Caux *et al.*, 1990; Backx *et al.*, 1991; Moore, 1991; Jacobsen *et al.*, 1992; Reid *et al.*, 1992). In these culture conditions the co-operation between TNFα and GM-CSF/IL-3 is critical for the development of DCs from CD34$^+$ HPC (Caux *et al.*, 1992; Reid *et al.*, 1992; Santiago-Schwarz *et al.*, 1992; Szabolcs *et al.*, 1995; Rosenzwajg *et al.*, 1996; Strunk *et al.*, 1996). Within 8 days in liquid cultures of CD34$^+$ HPC, addition of TNFα to GM-CSF yields a six- to eight-fold increase in cell number (Caux *et al.*, 1990, 1992). At day 12, 50–80% cells express CD1a (a molecule borne by LCs, and thymocytes) thus yielding 10×10^6 to 30×10^6 CD1a$^+$ cells from 10^6 CD34$^+$ HPC. Moreover, SCF or FLT3-L increase by three- to six-fold the yield of CD1a$^+$ cells (Siena *et al.*, 1995; Young *et al.*, 1995; unpublished data). These CD1a$^+$ cells are dendritic cells according to: (1) a typical morphology (Plate 8A–C); (2) a phenotype of DCs (expression of high MHC class II, CD4, CD40, CD54, CD58, CD80, CD86 and CD83, and a lack of CD64/FcγRI and CD35/CR1); (3) the presence of Birbeck granules (characteristic of LCs) in 20% of cells; and (4) a high capacity to stimulate proliferation of naive T cells and efficient presentation of soluble antigen to CD4$^+$ T-cell clones (Caux *et al.*, 1992, 1995, 1996b). CD1a$^+$ cells are CD45RO$^+$ and express the myeloid markers CD13 and CD33.

Recently, for cultures performed under serum-free conditions, TGFβ was shown to be required for the development of DCs with characteristics of LCs (Birbeck granules and Lag molecule) (Strobl *et al.*, 1996).

Using semi-solid cultures, DCs were shown to arise within single colonies together with monocytes/macrophages, suggesting the existence of a common precursor cell (Reid *et al.*, 1990, 1992; Santiago-Schwarz *et al.*, 1992).

Although GM-CSF or IL-3 (Caux *et al.*, 1996b), in association with TNFα or TNFβ, appear critical to support DC development from CD34$^+$ progenitors, other pathways of development have been reported. In this respect, *in vivo* injection of FLT3-L in mice induces a dramatic increase in

the number of mature DCs in lymphoid organs (Maraskovsky *et al.*, 1996). Also, CD40L induces a GM-CSF independent DC development from $CD34^+$ HPC (Florès-Romo *et al.*, 1997).

Identification of two pathways of dendritic cell development

While most cells are $CD1a^+CD14^-$ after 12 days of culture, at early time points (day 5–7) of the culture, two subsets of DC precursors, identified by the exclusive expression of CD1a and CD14, emerge independently (Caux *et al.*, 1996a) (Fig. 4). Both precursor subsets mature at day 12–14 into DCs with typical morphology (Plate 8D,E), phenotype (CD80, CD83, CD86, CD58, high HLA class II) and function. $CD1a^+$ precursors give rise to cells with Langerhans-cell characteristics (Birbeck granules, Lag antigen and *E*-cadherin). In contrast, the $CD14^+$ precursors mature into $CD1a^+$ DCs lacking Birbeck granules, *E*-cadherin and Lag antigen, but expressing CD2, CD9, CD68 and the coagulation factor XIIIa described in dermal dendritic cells. Interestingly, the $CD14^+$ precursors, but not the $CD1a^+$ precursors, represent bipotent cells that can be induced to differentiate, in response to M-CSF, into macrophage-like cells (Plate 8F), lacking accessory function for T cells. Furthermore, CD14- but not CD1a-derived DCs express IL-10 mRNA and protein (de Saint Vis *et al.*, 1998). These two pathways of development have been documented and characterized further by others. In particular, the commitment into either pathway has already occurred at the level of $CD34^+$ cells (Strunk *et al.*, 1997). Peripheral blood $CD34^+$, which express CLA (cutaneous lymphocyte associated

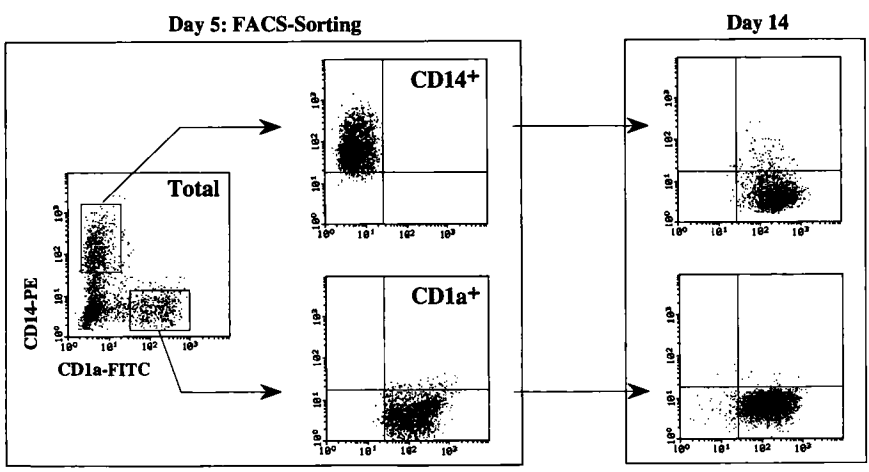


Figure 4. FACS-sorting of CD1a and CD14 DC precursors. Cord blood $CD34^+$ HPC were cultured for 5–6 days in the presence of SCF + GM-CSF + TNFα. The cells were then collected, processed for double staining using anti-CD14-PE and anti-CD1a-FITC, and FACS-sorted into $CD1a^+CD14^-$ and $CD14^+CD1a^-$ (left panel). Sorted cells were seeded in the presence of GM-CSF + TNFα (1×10^5 to 2×10^5 cells ml^{-1}) for 6–7 additional days, a last medium change being performed at day 10. At day 12, cells were reanalysed for CD1a and CD14 expression by double-colour fluorescence (right panel).

antigen), differentiate in response to GM-CSF plus TNFα into $CD1a^+$, Birbeck granule$^+$, Lag^+ Langerhans cells, while CLA^- progenitors differentiate into $CD1a^+$, Birbeck granule$^-$ and Lag^- interstitial DCs. While the two populations are equally potent in stimulating naive CD45RA cord blood T cells, each also displays specific activities (Caux *et al.*, 1997). In particular, CD14-derived DCs demonstrate a potent and long-lasting (from day 8 to day 13) antigen uptake activity (FITC–dextran or peroxidase) that is about 10-fold higher than that of $CD1a^+$ cells. The antigen capture is mediated exclusively by receptors for mannose polymers. The high efficiency of antigen capture of CD14-derived cells is co-regulated with the expression of non-specific esterase activity, a tracer of the lysosomal compartment. In contrast, the $CD1a^+$ population never expresses non-specific esterase activity. A striking difference between the two populations is the unique capacity of CD14-derived DCs to induce naive B cells to differentiate into IgM-secreting cells, in response to CD40 triggering and IL-2. Thus, while the two populations can allow T-cell priming, initiation of humoral responses might be preferentially regulated by the CD14-derived DCs.

Thus, different pathways of DC development might exist *in vivo*: (i) the Langerhans cell type, which might be mainly involved in cellular immune responses; and (ii) the CD14-derived DCs related to dermal DCs or circulating blood DCs, which may be more dedicated to the development of humoral immune responses.

Technical aspect

Collection and purification of cord blood CD34$^+$HPC

Umbilical cord blood samples are diluted by two-thirds in PBS and layered on Ficoll-Hypaque. The mononuclear cells are resuspended at 2×10^7 cells ml^{-1} in PBS/HS/EDTA and stained with anti-CD34 (Immu-133.3, 10 μg ml^{-1}, Immunotech Marseille, France). After 20–30 min at 4°C under gentle shaking, cells are washed three times with PBS/FCS/EDTA, and resuspended at 10^8 cells ml^{-1} in the presence of goat anti-mouse IgG coated microbeads (0.5 μl bead suspension per 10^6 cells, Miltenyi Biotec GmBH, Bergish Gladbach, Germany). After 20–30 min at 4°C under gentle shaking, cells are washed twice and isolation of $CD34^+$ progenitors is achieved using Minimacs separation columns (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) (Caux *et al.*, 1996a). Four 500-μl aliquots of the cell suspension (maximum $5 \times 10^7 ml^{-1}$, 200×10^6 total per column) are layered sequentially onto the top of the column fixed under the appropriate magnet. After four washes with 500 μl of medium the column is removed from the magnet and flushed twice with 2 ml RPMI complete medium. The isolated cells are 80–99% $CD34^+$ as judged by staining with anti-CD34 mAb. After purification $CD34^+$ cells are cryopreserved in 10% DMSO.

DC generation from CD34⁺HPC

Cultures are established in RPMI 1640 complete medium (see p. 516) supplemented with 100 ng ml^{-1} rhGM-CSF (specific activity 2×10^6 U mg^{-1}, Schering-Plough Research Institute, Kenilworth, NJ), 2.5 ng ml^{-1} rhTNFα (specific activity 2×10^7 U mg^{-1}, Genzyme, Boston, MA) and 25 ng ml^{-1} rhSCF (specific activity 4×10^5 U mg^{-1}, R&D Abington, UK) (Caux *et al.*, 1990, 1993). After thawing, $CD34^+$ cells are seeded for expansion in 25–75 cm^2 culture vessels (Linbro, Flow Laboratories, McLean, VA) at 2×10^4 cells ml^{-1}. Optimal conditions are maintained by splitting these cultures at day 4 with medium containing fresh GM-CSF and TNFα (cell concentration 1×10^5 to 3×10^5 cells ml^{-1}). For most experiments, cells are routinely collected after 5–6 days of culture for FACS sorting. Culture medium is supplemented with 2.5% AB^+ pooled human serum at the initiation of the cultures; by day 5–6 human serum is washed away (Caux *et al.*, 1996a).

Isolation of CD1a and CD14 DC precursors by FACS

After 5–6 days of culture in the presence of SCF, GM-CSF and TNFα, cells are collected and labelled with FITC-conjugated OKT6 (CD1a) (Ortho) and PE-conjugated Leu-M3 (CD14) (Becton-Dickinson & Co., Mountain View, CA), as described in Caux *et al.* (1996a). Cells are separated in $CD14^+CD1a^-$, $CD14^-CD1a^+$ fractions using a FACStarplus (see Fig. 4). All the procedures of staining and sorting are performed in the presence of 0.5 mM EDTA in order to avoid cell aggregation. Reanalysis of the sorted populations shows a purity higher than 98%, the other cells being immature myeloid cells (T cells could never be detected even using polymerase chain reaction (PCR) amplification of T-cell receptor components).

Sorted cells are seeded in the presence of GM-CSF + TNFα (1×10^5 to 2×10^5 cells ml^{-1}) for 6–7 additional days, a last medium change being performed at day 10. Cells are routinely collected between days 11 and 14. Adherent cells are eventually recovered using a 0.5 mM EDTA solution.

Different Pathways of Dendritic Cell Development

In vitro and *in vivo* studies in mice and humans indicate that several dendritic cell subsets may originate from at least three different progenitors (Fig. 5). A progenitor cell common to granulocytes, monocytes and dendritic cells (G-M-DC), identified in semi-solid medium, further differentiates into several lineage-specific precursors (for reviews see: Peters *et al.*, 1996; Young and Steinman, 1996; Cella *et al.*, 1997). $CD1a^+$ precursors give rise to Langerhans cells characterized by the

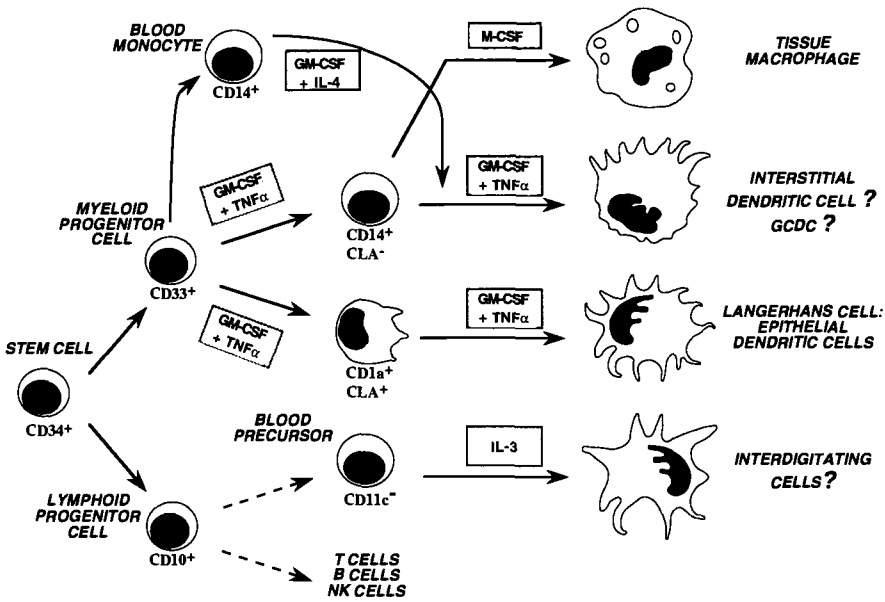


Figure 5. Different pathways of dendritic cell development. *In vitro* and *in vivo* studies in mice and humans suggest that several dendritic cell subsets may originate from at least three different progenitors. A progenitor cell common to granulocytes, monocytes and dendritic cells (myeloid progenitor, $CD33^+$), identified in semi-solid medium, further differentiates into several lineage-specific precursors. DC-specific precursors (CLA^+CD1a^+) lead to Langerhans cell type DCs. In the presence of GM-CSF, CLA^-CD14^+ lead to interstitial type DCs that could be related to blood or tonsil $CD11c^+$ DC (GCDC). The same precursor CLA^-CD14^+ differentiates into macrophages in the presence of M-CSF. Finally, the existence of a third progenitor for thymic DCs has also been demonstrated in mice. This progenitor displays T and B lymphoid, but no myeloid, differentiation potential. In human, this progenitor ($CD10^+$?) might lead to $CD11c^-$ DC precursor identified in blood and tonsils, which would further differentiate into a subset of IDCs.

expression of Birbeck granules, the Lag antigen and *E*-cadherin. In contrast, the $CD14^+$ progenitors mature into $CD1a^+$ DCs lacking Langerhans cell antigens but expressing CD11b, CD9, CD68 and the coagulation factor XIIIa described in dermal DCs. Interestingly, the $CD14^+$ precursors, but not the $CD1a^+$ precursors, represent bipotent cells that can be induced to differentiate, in response to M-CSF, into macrophage-like cells.

Following maturation signal, monocyte-derived DCs display a full dendritic cell phenotype and may be closest to dermal DCs or CD14 precursors derived DCs in view of the lack of Birbeck granules and expression of CD11b and CD68. Indeed, it is tempting to speculate that dermal DCs may originate from monocytes that have entered tissues and encountered IL-4 or IL-13 released by tissular mast cells. Like $CD14^+$ precursors, monocytes can differentiate into macrophages in presence of M-CSF (Chapuis *et al.*, 1997). The Langerhans cell type might be mainly involved

in cellular immune responses, while the monocyte derived DCs could be involved in humoral immune responses.

In mice, a thorough analysis of cellular populations within the mouse thymus has permitted the identification of progenitors that can differentiate into either T cells or DCs when injected in thymus lobes or into B cells following homing into the spleen after intravenous injection (Ardavin *et al.*, 1994; Saunders *et al.*, 1996; Wu *et al.*, 1996). In no case have these progenitors been found to differentiate into the myeloid pathway. Such T-B-DC progenitors might have been identified in humans (Galy *et al.*, 1995; Res *et al.*, 1996). The function of this cell population remains undetermined.

Recent experiments with mice whose Rel-B, TGFβ1 or Ikaros gene have been disrupted illustrate further the different origin/relationship of dendritic cell subsets. Rel-B$^{-/-}$ animals display epidermal LCs but no DCs in their thymus and spleen (Burkly *et al.*, 1995; Weih *et al.*, 1995). In contrast, TGFβ$^{-/-}$ mice lack Langerhans cells but display CD11c^{+} DC in lymph nodes (Borkowski *et al.*, 1996). Ikaros$^{-/-}$ lacking T, B and NK cells display a deficiency in CD11c^{+} splenic DCs. In contrast epidermal Langerhans cells and myeloid lineages (granulocytes, monocytes) are not affected (Wang *et al.*, 1996).

◆◆◆◆◆◆ FUNCTIONAL CHARACTERIZATION OF DENDRITIC CELLS

Antigen Uptake

Characteristic features

Although described as professional APCs, DCs were considered, until recently, as displaying poor endocytic and phagocytic capacities (for reviews see: Steinman and Swanson, 1995; Austyn, 1996). Yet, LCs were shown to display all steps of receptor-mediated endocytosis (Hanau *et al.*, 1987), and to phagocytose particles of relatively large size such as latex beads (Matsuno *et al.*, 1996), viruses (Barfoot *et al.*, 1989), bacteria (Schuler *et al.*, 1983; Reis e Sousa *et al.*, 1993) and intracellular parasites such as *Leishmania major* (Moll, 1993). DCs can concentrate extracellular solute into vacuoles through macropinocytosis (Sallusto *et al.*, 1995). Antigen uptake by afferent lymph DCs can also occur in the form of immune complexes (Harkiss *et al.*, 1990). Finally, DCs express lectins such as the mannose receptor and DEC205, with multiple lectin-like domains (Jiang *et al.*, 1995; Sallusto *et al.*, 1995). Such molecules mediate through specific glycan recognition efficient antigen uptake and delivery to MHC class II compartment allowing optimal antigen presentation to CD4 T-cell clones. These molecules are likely to contribute to the uptake of bacterial antigens that display protein glycosylation patterns different from mammals, and thus might represent a first line of discrimination between self and non-self.

Quantitation of endocytosis using FITC dextran capture and flow cytometry

The method described below is after Sallusto *et al.* (1995). DCs are resuspended at $5 \times 10^5 ml^{-1}$ in 10% FBS medium buffered with 25 mM HEPES at 37°C in a water bath. FITC–dextran is added at the final concentration of 0.1 mg ml^{-1} and for 15 (5–60) min to 10^5 cells in 5-ml polypropylene tubes (Falcon, Becton Dickinson). The cells are washed four times with cold PBS containing 1% FBS and 0.01% NaN_3. To characterize the endocytosing cell population further, double staining may be done using anti-CD1a-PE (Coulter, Hialeah, FL) or anti-CD14-PE (Becton Dickinson) or any other PE-coupled mAb. To analyse the fate of endocytosed material, cells may be pulsed at 37°C; washed four times in cold medium, and recultured at 37°C for different times in marker-free medium. After staining, cells are analysed using a FACS. When the results are expressed as MFI, the background (cells pulsed with FITC–dextran at 4°C) is subtracted.

Quantitation of endocytosis by horseradish peroxidase capture

The method described below is after Sallusto *et al.* (1995). DCs are resuspended at $5 \times 10^5 ml^{-1}$ in 10% FBS medium buffered with 25 mM HEPES at 37°C in a water bath. Horseradish peroxidase (HRP, Sigma) is added to 10^5 cells at a final concentration of 0.1 μg ml^{-1} to 0.1 mg ml^{-1} for 15 min. The cells are washed four times with cold PBS containing 1% FBS and 0.01% NaN_3, lysed with 0.05% Triton X-100 in 10 mM Tris buffer, pH 7.4, for 30 min. After centrifugation (10 min, 600*g*), the enzymatic activity of the lysate is measured using ABTS (2,2-azinobis-3-ethylbenthiazoline-6 sulfonic acid) as substrate at 1 mg ml^{-1} in buffer (0.1 M citric acid, 0.2 M disodium hydrogenophosphate) supplemented with 0.1 μl ml^{-1} H_2O_2 (30%). The OD is read at 420 nm with reference to a standard curve.

Interactions between Dendritic Cells and T Cells

Characteristic features

The capacity of DCs in priming T cells *in vivo* has been demonstrated directly in cell-transfer experiments. Thus, upon reinjection into foot pad or blood or upon intratracheal instillation, mouse DCs pulsed *in vitro* with protein antigen induced MHC restricted, antigen-specific T-cell responses (Inaba *et al.*, 1990a,b; Havenith *et al.*, 1993; Levin *et al.*, 1993; Liu and MacPherson, 1993). Also, mouse DCs pulsed with HIV envelope derived or tumour-restricted peptides have been shown to induce $CD8^+$ cytotoxic responses *in vivo* and tumour clearance (Takahashi *et al.*, 1993; Flamand *et al.*, 1994; Mayordomo *et al.*, 1995;

Boczkowski *et al.*, 1996; Celluzzi *et al.*, 1996; Paglia *et al.*, 1996; Zitvogel *et al.*, 1996).

Recently, the availability of mice expressing transgenic T-cell receptors has allowed the analysis of the capacity of DCs to induce a primary antigen-specific T-cell response to soluble antigens *in vitro*. In such systems DCs appear to be 100- to 300-fold more efficient than any other APC (Croft *et al.*, 1992; Macatonia *et al.*, 1993).

The most commonly used functional assessment of DCs is their remarkable ability to drive the allogeneic mixed lymphocyte reaction (MLR). DCs are about 100-fold more efficient than any other APC population, including B cells and monocytes (Steinman and Witmer, 1978; Crow and Kunkel, 1982; Van Voorhis *et al.*, 1983). DCs can also stimulate allogeneic $CD8^+$ T cells, although higher APC numbers are required (Inaba *et al.*, 1987; Young and Steinman, 1990). The use of superantigens has further demonstrated the efficiency of DCs in primary T-cell activation (Bhardwaj *et al.*, 1992, 1993, 1994b).

DCs generated *in vitro* from $CD34^+$ HPC induce a strong proliferation of allogeneic naive $CD4^+$ T cells (Plate 8G,H) and of syngeneic naive $CD4^+$ T cells in the presence of a low concentration of superantigens (Caux *et al.*, 1992, 1995). The proliferation of allogeneic $CD8^+$ T cells is weaker than that of $CD4^+$ T cells when cultured with DCs alone, but reaches comparable levels in the presence of cytokines such as IL-2, IL-4 or IL-7. Accordingly, allospecific $CD4^+$ or $CD8^+$ T-cell lines can be generated by repeated culturing of T cells on DCs, and the $CD8^+$ T-cell lines display a high cytotoxic potential in a MHC-restricted manner (Caux *et al.*, 1995; Wettendorff *et al.*, 1995). DCs generated from $CD34^+$ HPCs are also able to present soluble antigen to MHC-matched tetanus toxoid-specific T-cell clones (Caux *et al.*, 1995).

Technical aspect

T-cell proliferation assay

After 12 days of culture, $CD34^+$ HPC-derived cells are used, after 30-Gy irradiation, as stimulator cells for: (i) resting allogeneic adult or cord blood T cells (CD3, CD4, CD8, CD45RA, 2.5×10^4 per well); (ii) resting syngeneic cord blood $CD4^+$ T cells (2.5×10^4 per well) in the presence or absence of superantigen; and (iii) syngeneic $CD4^+$ T cells from specific T-cell clones (10^4 per well) (Caux *et al.*, 1994a,c). Naive T cells are purified by negative selection using mAbs and magnetic beads. Stimulator cells (6×10^4 to 3×10^4) are added to the T cells in 96-well round-bottomed microtest tissue-culture plates (Nunc, Roskilde, Denmark). Cultures last 5 days for resting $CD4^+$ T cells and 3 days for T-cell clones. After incubation, cells are pulsed with 1 μCi of [^{3}H]TdR (specific activity 25 Ci mmol^{-1}) per well, for the last 8 h, harvested and counted. [^{3}H]TdR uptake by stimulator cells alone is always below 100 cpm.

Role of CD40/CD40L in DC/T Cell Interactions

Characteristic features

The CD40 antigen, which is of critical importance in T-cell dependent B-cell growth, differentiation and isotype switch, is also functional on other cell types (Banchereau *et al.*, 1994). In particular, CD40 is expressed on LCs, blood DCs, interdigitating cells as well as *in vitro* generated DCs (Hart and McKenzie, 1988; Lenz *et al.*, 1993; Nestle *et al.*, 1993; O'Doherty *et al.*, 1993, 1994; Caux *et al.*, 1994b). Transferring *in vitro* generated DCs into a medium devoid of GM-CSF results in their prompt death unless their CD40 antigen is engaged. In addition, CD40 triggering induces changes in morphology and phenotype. Thus, CD40-activated DCs upregulate accessory molecules such as CD58, CD80 and CD86 and secrete cytokines (TNFα, IL-8 and MIP1α). CD40 engagement also turns on DC maturation, as illustrated by up-regulation of CD25 and down-regulation of CD1a that, respectively, appear and disappear on LC/veiled cells when they enter secondary lymphoid organs to become interdigitating dendritic cells (Romani *et al.*, 1989; Larsen *et al.*, 1990). As T cells that are activated by DC up-regulate CD40-L (Caux *et al.*, 1994b), it is likely that CD40 activation of DCs represents an important physiological interaction between DCs and T cells. The role of CD40 engagement on DC activation/maturation has also been clearly demonstrated using monocyte-derived DCs. CD40 triggering on GM-CSF+IL-4 -derived DCs induces upregulation of co-stimulatory molecules, translocation of MHC class II from intracellular compartment to cell surface, and, importantly, secretion of IL-12 (Cella *et al.*, 1996; Koch *et al.*, 1996). So far, CD40 engagement appears to be the only stimulus allowing IL-12 production by DCs. The production of IL-12 by DCs following interaction with T cells results in the production of IFN-γ by the primed T cells (Macatonia *et al.*, 1995; unpublished data).

In keeping with this, T-cell alloreaction induced by blood DCs (Zhou and Tedder, 1995) and epidermal LCs (Péguet-Navarro *et al.*, 1995) is inhibited by antibodies interrupting CD40/CD40L interactions. Furthermore, patients that have a genetically altered non-functional CD40-L, present a major T-cell defect (impaired IL-12/IFN-γ production), as shown by frequent *Pneumocystis carinii* infections (Notarangelo *et al.*, 1992; Grewal *et al.*, 1995; Kamanaka *et al.*, 1996). Finally, *in vivo* administration of CD40/CD40-L antagonist has been shown to block the development of T-cell dependent collagen induced arthritis (Durie *et al.*, 1993) as well as graft-versus-host disease (Durie *et al.*, 1994). Taken together, these data can be interpreted as reflecting the interruption of a CD40/CD40-L interaction between T cells and DCs, rather than between T cells and B cells. A cell-specific disruption of the CD40 gene would allow clarification of the role of CD40 in the various cell types.

Technical aspect

CD40 activation

L cells transfected with human CD40 ligand (Garrone *et al.*, 1995) (CD40L-L cells) are used to induce CD40 triggering on D-Lc. CD32 transfected L cells (CD32 L cells) are used for control cultures. L cells (5×10^4) are seeded together with 4×10^5 cells (either CD34 derived GM-CSF + TNFα DCs, or monocyte-derived DCs) per well (24-well culture plate, Linbro) in the presence or absence of GM-CSF (100 ng ml^{-1}) in 1-ml culture medium and cultured for 2–4 days. Cell survival is monitored by enumeration of cells excluding Trypan blue. For determination of cytokine production, supernatants are recovered after 48 h. For phenotypic and morphological analysis, cells are recovered after 4 days of culture.

Interactions between Dendritic Cells and B Cells

Characteristics features

Although the T-cell-dependent primary B-cell activation is known to be dependent on DCs (Inaba *et al.*, 1983, 1984; Francotte and Urbain, 1985; Inaba and Steinman, 1985; Sornasse *et al.*, 1992; Flamand *et al.*, 1994) and to occur in the extrafollicular area of secondary lymphoid organs, little information is presently available regarding the potential interactions between DCs and B cells. In a system, where the T-cell signal is provided by CD40-L transfected L cells, $CD34^+$ HPC-derived DCs have been found directly to modulate B cell growth and differentiation (Dubois *et al.*, 1997). In particular, DCs induce a three- to six-fold enhancement of CD40-L dependent B-cell proliferation in the absence of exogenous cytokines (Plate 8I). Furthermore, DCs considerably enhance (10- to 100-fold increase) the secretion of IgG, IgA and IgM by CD40-activated memory B cells, in the absence of exogenous cytokines. Importantly, in the presence of DCs, naive $sIgD^+$ B cells produce, in response to IL-2, large amounts of IgM. This latter effect is dependent on the release by DCs of soluble mediators after CD40 engagement. In addition, in the presence of IL-10, DCs stimulate CD40-activated naive $sIgD^+$ B cells to express surface IgA and secrete large amounts of both IgA subclasses (Fayette *et al.*, 1997).

Technical aspect

Co-culture of B cells and DC

10^4 $sIgD^+$ B lymphocytes, purified by preparative magnetic cell sorter (Miltenyi Biotec) (Defrance *et al.*, 1992), are seeded together with 2.5×10^3 irradiated transfected L cells (75 Gy), with or without irradiated (30 Gy) DCs (3×10^3 per well) in 96-well culture plates

(Nunc), as described by Dubois *et al.* (1997). For measurement of proliferation, cells are pulsed, after 5 days of incubation, with 1 μCi of [^{3}H]TdR (specific activity 25 Ci mmol^{-1}) per well, for the last 8 h, harvested and counted. [^{3}H]TdR uptake by stimulator cells alone is always below 100 cpm. For determination of Ig production, supernatants are harvested after 15 days and used for indirect ELISA (Defrance *et al.*, 1988).

◆◆◆◆◆◆ POTENTIAL PHYSIOLOGICAL RELEVANCE

DCs form a heterogeneous family of cells with characteristic features. Their heterogeneity in terms of lineages and functions is not yet fully elucidated. At least three independent pathways yielding different DC populations have been identified in human and mouse (see Fig. 5). In humans, two pathways of development yielding DCs of myeloid origin have been documented. Langerhans cells can be generated from CD34$^+$ HPCs when cultured in presence of GM-CSF + TNFα. Another population of DCs can be generated from CD34$^+$ HPCs under the same culture conditions. However, this population lacks the characteristics of Langerhans cells and is probably related to the monocyte-derived DCs. Although this cell type displays phenotypic similarity with interstitial DCs and CD11c$^+$ circulating DCs or tonsil germinal centre DCs, their *in vivo* counterpart is not clearly established. Based on the effect of CD14-derived DCs (also borne by monocyte-derived DCs) on naive B cell differentiation it is tempting to speculate that this population might be related to CD11c$^+$ DCs located within B-cell follicules in tonsils.

Thus different pathways of DC development might exist *in vivo*. The Langerhans cell type might be mainly involved in cellular immune responses, an idea which is supported by the involvement of LCs in delayed-type hypersensitivity reactions observed after hapten application on the epidermis (Sullivan *et al.*, 1986; Macatonia *et al.*, 1987; Larsen *et al.*, 1990). In contrast, CD14-derived DCs related to monocyte-derived DCs potentially located into tissues such as dermis or blood, might, after antigen capture, migrate through the blood (or lymph) stream into either the T-cell-rich area and/or the B-cell follicles, where they could be involved in the regulation of primary B-cell responses.

In mice, the lymphoid origin of thymic DCs and one subset of splenic DCs has been demonstrated by Shortman and collaborators. Although this pathway has not yet been clearly demonstrated in humans, the CD11c$^-$CD13$^-$DC precursors identified in blood and in tonsils might be related to mouse lymphoid DCs. The work of Shortman (Kronin *et al.*, 1996; Süss and Shortman, 1996) suggests that this lymphoid population might be involved in the negative regulation of T-cell activation, playing a role in the maintenance of peripheral tolerance.

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2 Isolation of T Cells and Establishment of T-cell Lines and Clones

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Introduction
Isolation of mononuclear cells
Separation of T and non-T cells from mononuclear cells
Separation of T-cell subsets
Establishment of T-cell lines
Establishment of T-cell clones

INTRODUCTION

The study of human T cells is best performed using purified cells, since the presence of other cell types may have indirect effects on T-cell function. However, for any kind of functional assay on T-cell specificity, antigen-presenting cells are necessary. In this chapter we describe the basic procedures for isolating mononuclear cell fractions containing monocytes and T and B lymphocytes from different sources, i.e. human peripheral blood, body fluids or diseased tissues. Other experimental methods described involve the separation of T cells and T-cell subsets from mononuclear cell populations, and the generation of T-cell lines and T-cell clones with specificity for bacterial antigens.

ISOLATION OF MONONUCLEAR CELLS

Mononuclear Cell Isolation from Peripheral Blood

The mononuclear cell fraction containing monocytes and T and B lymphocytes is separated from polymorphonuclear and red blood cells by density gradient centrifugation.

METHODS IN MICROBIOLOGY, VOLUME 25
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Equipment and reagents

- Suppl. RPMI-1640 medium: RPMI-1640 (Life Technologies Inc., Gaithersburg, MD) containing 2 mM L-glutamine (Biochrom Seromed, Berlin, Germany), 25mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES) (Biochrom), 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin (Pen-Strep, Biochrom).
- Fetal calf serum (FCS) (e.g. Life Technologies Inc.) which has been inactivated by heat (56°C, 30 minutes) before use.
- Heat-inactivated human serum, blood group AB (HUS, obtained from a local blood transfusion centre).
- Ficoll-Hypaque (Histopaque-1077, Sigma).
- 50- and 15-ml conical centrifuge tubes (e.g. Greiner, Nürtingen, Germany).
- Temperature-controlled centrifuge with GH-3.7 horizontal rotor (e.g. Heraeus or Beckman).
- Trypan blue, haemocytometer.

Procedure

(Modified from Boyum, 1968.)

Peripheral blood is collected in sterile heparinized tubes. Heparinized whole blood (10 ml) is mixed with 15 ml suppl. RPMI-1640. The mixture is carefully layered over 15 ml of Ficoll-Hypaque in a 50-ml conical centrifuge tube. Spin for 20 min at 2000 rpm (900*g*, 4°C). The layer between Ficoll and the upper layer (containing RPMI-1640 and serum) contains the mononuclear cell (MNC) fraction (Fig. 1). Using a pipette remove 80% of the upper layer and recover the interface (MNC) layer. Transfer the latter to a new 50-ml conical tube, and fill the tube with suppl. RPMI-1640/5% fetal calf serum (FCS) and centrifuge for 10 min at 1300 rpm (400*g*, 18°C). After the supernatant has been removed, the MNC pellet is resuspended in suppl. RPMI-1640/5% FCS, and the wash is repeated twice. For the last wash, 15-ml conical tubes can be used. Finally, the cells are resuspended in 1 ml suppl. RPMI-1640/10% heat-inactivated human AB serum (HUS).

Counting and markers of cell death

Cell suspension (20 µl) is diluted with 20 µl 0.5% aqueous Trypan blue. The stained (dead) and non-stained (viable) cells are counted in a haemocytometer.

Isolation of Mononuclear Cells from Synovial Fluid or Synovial Membrane

There is growing evidence that bacteria and other micro-organisms play an important role in the aetiopathogenesis of several inflammatory rheumatic diseases such as reactive arthritis, rheumatic fever, Lyme disease and the so-called HLA-B27 associated seronegative spondyl-

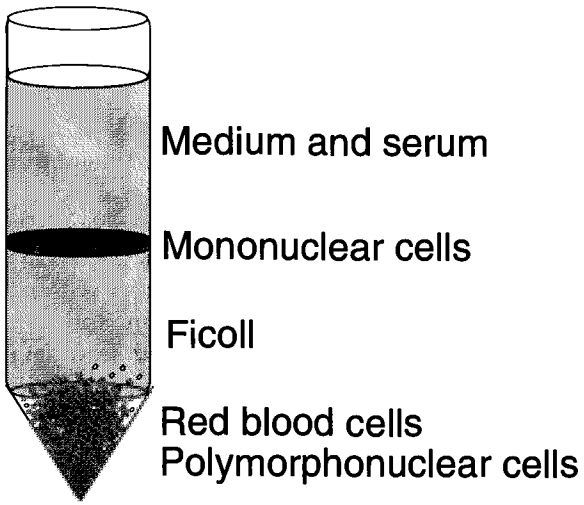

Figure 1. Separation of mononuclear cells (MNCs) by Ficoll–Hypaque density gradient centrifugation.

arthropathies. Furthermore, T cells appear to play a major role in the development, maintenance and also resolution of these forms of bacteria-associated arthritides. Recent developments in understanding the processes involved in T-cell activation now allow us to examine the synovial fluid and synovial membrane T-cell responses to 'arthritogenic' micro-organisms in terms of antigen specificity, epitope identification, cytokine secretion patterns, cytotoxicity and HLA-restriction (Hermann, 1993). The relative importance of the $\alpha\beta$-TCR$^+$ CD4$^+$ and CD8$^+$ and the $\gamma\delta$-TCR$^+$ T-cell populations in the pathogenesis of these inflammatory rheumatic diseases has yet to be determined.

Equipment and reagents

- Heparinized synovial fluid (obtained as part of the routine diagnostic and therapeutic management), synovial tissue from surgical synovectomies.
- Sterile PBS; suppl. RPMI-1640; heat-inactivated FCS (Life Technologies Inc.); heat-inactivated HUS, HEPES (Biochrom); collagenase (Type CLS II, Biochrom); deoxyribonuclease (Dnase Type II, Sigma); trypsin–EDTA mixture (0.05% w/v trypsin, 0.02%w/v EDTA, e.g. ICN Pharmaceuticals, Costa Mesa, CA), Ficoll–Hypaque (Histopaque-1077, Sigma).
- Sterile scissors; 50-ml glass spinner flask; sterile steel sieves (mesh 80); sterile Petri dishes; 50-ml conical centrifuge tubes (e.g. Greiner); temperature-controlled centrifuge; humidified 37°C, 5% CO_2 incubator (e.g. Heraeus).

Procedure

(Abrahamsen *et al.*, 1975; Burmester *et al.*, 1981; Klareskog *et al.*, 1982.)

- *Synovial fluid derived MNCs.* These are isolated by Ficoll-Hypaque density-gradient sedimentation in exactly the same way as described for peripheral blood MNC (see page 540).
- *Isolation of synovial membrane MNCs.* Synovial tissues from routine synovectomies are collected in sterile PBS, stored at 4°C and transported to the laboratory within 2 h. Fat and fibrous material are carefully removed from the synovial lining layer. The resulting synovium is minced into small pieces with sterile scissors, suspended in balanced salt solution containing 20 mM HEPES, pH 7.4, 0.2 mg ml^{-1} collagenase, and 0.15 mg ml^{-1} deoxyribonuclease, and stirred for 1 h at 37°C in a 50-ml glass spinner flask. The digested mixture is filtered through a sterile steel sieve (mesh 80) to remove large clumps of debris. The resulting single-cell suspension is further cleared from dead cells and subcellular material by standard Ficoll-Hypaque centrifugation, as described above (see page 540), washed and suspended in suppl. RPMI-1640/10% FCS.
- *Preparation of adherent synovial cells.* The purified cell suspension is seeded onto sterile Petri dishes at an approximate cell concentration of 5×10^5 cells ml^{-1}. After 45 min of incubation at 37°C, 5% CO_2, the non-adherent cells are removed from the adherent cell layer by gently washing the Petri dishes three times with warm RPMI-1640. Cells adhering to the Petri dishes are further incubated in suppl. RPMI-1640/10% FCS overnight, and then washed twice with PBS containing 0.02% EDTA and subjected to treatment with a trypsin–EDTA mixture (0.05%w/v trypsin, 0.02%w/v EDTA) at 37°C until virtually all cells are in suspension as visualized by phase-contrast microscopy (usually after 15–30 min). This cell suspension is washed twice in suppl. RPMI-1640/5% FCS and finally resuspended in suppl. RPMI-1640/10% HUS. It has been shown that this preparation of adherent synovial cells contains type-A synoviocytes (macrophage-like synovial cells) and type-B synoviocytes (fibroblast-like synovial cells). The cells can be used as stimulator cells (irradiated antigen presenting cells with soluble bacterial antigen, or as stimulators in an autologous or allogeneic mixed leucocyte culture reaction). Furthermore, they can be further propagated and passaged *in vitro*, and characterized by immunofluorescence or immunoperoxidase staining, or by the secretion of cytokines and enzymes into the supernatant.
- *Preparation of synovial membrane derived T-lymphocytes.* The non-adherent cells obtained as described above are washed twice and further fractionated into T and non-T non-adherent cells by separating the cells that form spontaneous rosettes with neuraminidase-treated sheep red blood cells (see page 548).

Mononuclear Cell Isolation from Gut Mucosa

In humans, access to gut-associated lymphoid tissue (GALT) is usually obtained through intestinal resections or procurement of biopsy specimens. The decision to use either one of these will be influenced by the experimental setting. Thus if early or focal intestinal lesions are to be studied it is necessary to use biopsy specimens, whereas if the aim is to obtain large numbers of cells surgical resections are needed.

Another important question that needs to be settled before starting an isolation procedure, is to define the cell population of interest. This decision will be aided by the knowledge that lymphoid cells within the GALT reside in several morphologically and functionally distinct compartments (Fig. 2). These include an afferent or inductive limb constituted by cells localized in organized lymphoid tissues in the form of Peyer's patches (PPs), mesenteric lymph nodes (MLNs) and the appendix, as well as single follicles beneath the intestinal epithelium. PPs are a sampling site for intestinal antigens, and play a major role in the induction of a mucosal immune response. Lymphocytes primed within the PPs enter the circulation via the mesenteric lymph node and thoracic duct. From here, they recirculate to less organized lymphoid effector regions within the lamina propria and intestinal epithelium. T lymphocytes within the lamina propria (LP-T) secrete B cell helper factors upon specific stimulation and perform important effector functions. They are mostly $CD4^+$ (60–70%) and $\alpha\beta$-TCR^+ (95%), and display a memory phenotype. In contrast, intraepithelial lymphocytes (IELs) are located between the epithelial cells. They are mostly $CD8^+$ T cells, and display a variety of phenotypic differences compared to lamina propria or peripheral blood T cells. IELs may be a first line of defence, but their physiological function is still being investigated.

As most groups seem to be interested in studying effector T cells, we describe here methods for isolating LPMCs and IELs from intestinal resections. The procedure described is a modification of previously described procedures (Bull and Bookman, 1977; Fiocchi *et al.*, 1979). Further modifications for isolation of IELs have been reported (Cerf-Bensussan *et al.*, 1985; Lundquist *et al.*, 1992). The procedure used to isolate mononuclear cells from biopsy specimen follows the same principle. If pure separations of LPMCs or IELs are important, cross-contamination may be a problem with biopsy specimens and can be controlled by using FACS analysis.

Although in some experiments it may be necessary to isolate immune cells from the afferent limb of the intestinal immune system (i.e. cells localized in organized lymphoid tissue in the form of PPs and MLNs) a detailed description of the methods used is not given here. However, for those who are interested in the isolation of these cells, it should be stated that lymphocytes from PPs, which extend through the mucosa and into the submucossa, can be isolated from small-bowel resections or ileal biopsy specimens (MacDonald *et al.*, 1987) using collagenase digestion. Lymphocytes from MLNs, which can be obtained from the mesentery of surgically resected specimens, can be isolated by teasing through a steel sieve (mesh 80) after MLNs have been cleaned from fat and vessels. In general, isolation of mononuclear cells from MLNs is no different from isolation of MNCs from other lymph nodes.

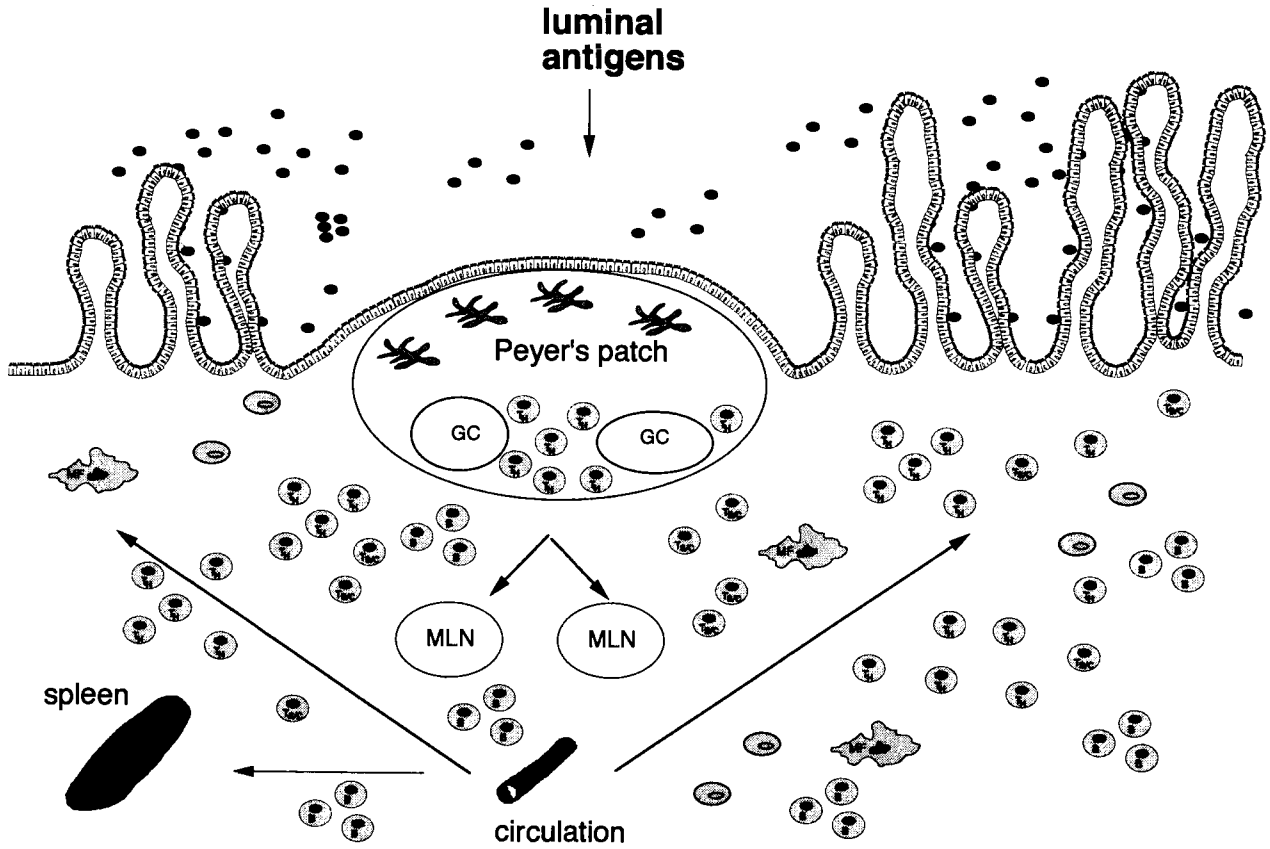

Figure 2. Components of the gastrointestinal immune system, T_H, T helper cell; Ts/c, T suppressor/cytotoxic cell; B, B cell; IEL, intraepithelial lymphocytes; GC, germinal centre; MLN, mucosal lymph node.

Equipment and reagents: isolation of lamina propria lymphocytes from surgical resections

- Fresh mucosal tissue from surgical resection.
- PBS; Hanks w/o Ca/Mg; RPMI-1640 (Biochrom), HEPES (Biochrom); penicillin/streptomycin (Pen-Strep, Biochrom); gentamycin (Life Technologies Inc.); amphotericin B (Sigma); DTT (Sigma); EDTA (Sigma); 2-ME (Sigma); collagenase Typ IV (Sigma); Dnase type II (Sigma); Ficoll-Hypaque (Histopaque-1077, Sigma); Percoll (Pharmacia Biotech Inc., Piscataway, NY).
- Surgical scissors; nylon strainer (100 µm); sterile Erlenmeyer flasks; sterile steel sieves (mesh 80); magnetic stirrer (IKAMAG); 50-ml conical centrifuge tubes (e.g. Greiner); temperature-controlled, refrigerated centrifuge (e.g. Heraeus).

Procedures

1. Fresh mucosal tissue from surgical resections is washed with sufficient amounts of PBS (500 ml) to remove gross contaminants and the mucosa is dissected from the underlying muscular layer with scissors.
2. To remove mucus, tissue is then incubated in 150 ml isolation solution A (Hanks w/o Ca/Mg 500 ml containing 1× Pen-Strep, 500 µl gentamycin, amphotericin B 1.25 mg, 5 ml L-glutamine, 25 mM HEPES, 500 mg DTT) in a sterile Erlenmeyer flask with a magnetic stirrer for 20 min at 200 rpm and 37°C. To increase yield, this step is repeated twice or until the tissue surface remains free of mucus. Before proceeding to step 3 the supernatant is discarded and the tissue is rinsed as in step 1.
3. To remove epithelial cells, tissue is incubated in 150 ml isolation solution B (Hanks w/o Ca/Mg 500 ml containing 1× Pen-Strep, 500 ml gentamycin, 5 ml L-glutamine, amphotericin B 1.25 mg, 12.5 mM HEPES, 25 µl 2-ME, 2.5 ml EDTA 0.2 M) in a sterile Erlenmeyer flask with a magnetic stirrer for 30 min at 200 rpm and 37°C. Supernatant is saved for isolation of IELs, and tissue is rinsed vigorously as in step 1. Step 3 is repeated three times, or until clear supernatant indicates that all epithelial cells have been removed.
4. To liberate LPMCs, the tissue is cut into 1-mm pieces using sharp surgical scissors, and the pieces incubated in approximately 150 ml prewarmed enzyme solution (500 ml RPMI-1640 containing 5 ml Pen-Strep, 500 µl gentamycin, 5 ml L-glutamine, 25 mM HEPES, 50 mg collagenase type IV, and 50 mg DNase) for 60 min at 200 rpm and 37°C.
5. Supernatant containing the LPMCs is poured off and RPMI-1640 is added 1:1 to dilute the enzymes. Tissue is resubmitted to step 4, while keeping isolated cells on ice.
6. To collect the cells, supernatants are transferred to 50-ml conical tubes, and centrifuged for 10 min at 1300 rpm (400*g*). Sedimented

LPMCs are resuspended in 100 ml RPMI-1640 and passed through a nylon strainer to remove clumps and residual debris.

7. To isolate the mononuclear cells, 25-ml aliquots of cells are layered onto each of 4–8 Ficoll-Hypaque gradients for density sedimentation (1450 rpm, 15 min). If necessary, recovered LPMCs can be stored overnight on ice in 25–50 ml suppl. RPMI-1640.

An experienced worker can isolate LPMCs from intestinal resections within 8–10 h.

Pitfalls

Isolation of LPMCs from intestinal resections may be complicated due to low cell viability, low cell yield or cross-contamination with other cell types (epithelial cells, IELs). In addition, it is a common experience that it is difficult to standardize the procedure, and the length of the isolation procedure certainly increases the risk of technical errors. If low cell yield is a problem, different causes should be considered. First, resections that contain increased ratios of collagenous tissue (stenosed or bulky resections from Crohn's disease) may contain, and thus yield, low numbers of LPMCs. Second, low cell yield may be due to incomplete dissection of the mucosa or incomplete removal of fat and mucus from the tissue, producing sticky clumps that entrap LPMCs. Furthermore, cell yield and cell viability are often inversely correlated. Thus, increasing the digestion time and the amounts of enzymes may give better cell yields at the cost of decreased cell viability, and vice versa. Other enzymes than collagenase (e.g. DISPASE) have been tested, but collagenase seems to give the best yield of viable cells with unaltered expression of cell surface molecules. Fortunately, bacterial contamination is rarely a problem because of the many washing steps and Percoll fractionations involved in the protocol. When beginning to isolate LPMCs, significant contamination with other cell types, especially epithelial cells and IELs, should be excluded by FACS analysis.

Intraepithelial lymphocytes

Equipment and reagents

- Fresh mucosal tissue from surgical resection.
- PBS; Hanks w/o Ca/Mg; RPMI-1640 (Biochrom); HEPES (Biochrom); Pen-Strep (Biochrom); gentamycin (Gibco Life Technologies Inc.); amphotericin B (Sigma); DTT (Sigma); EDTA (Sigma); 2-ME (Sigma); Percoll (Pharmacia).
- Surgical scissors; sterile Erlenmeyer flasks; magnetic stirrer; 50-ml centrifuge tube; temperature-controlled, refrigerated centrifuge (Heraeus).

Procedures

The first three steps for isolating IELs are identical to those described above for isolation of LPMC. Following incubation with isolation solution B, supernatants containing the IELs are diluted 1:1 in RPMI-1640. The cell suspension is transferred via a pipette to 50-ml centrifuge tubes. Epithelial cells are then collected from the solution by centrifugation for 10 min at 1300 rpm. After resuspension in suppl. RPMI-1640, cells are layered onto Percoll gradients containing 10-ml layers of 70%, 40%, 20% and 10% Percoll. After centrifugation for 30 min at 1600 rpm and 4°C, IELs can be collected between the 70% and 40% steps, whereas epithelial cells will sediment at the interface between the 40% and 20% Percoll steps.

An experienced worker can isolate IELs from intestinal resections within 4 h.

Pitfalls

When beginning to isolate IELs, significant contamination with other cell types, especially LPMCs, should be excluded by FACS analysis demonstrating low numbers of $CD20^+$ and $CD14^+$ cells. If purity from epithelial cells is important, modifications of the procedure may be necessary (Lundquist *et al.*, 1992). When standardizing the isolation procedure, the source of IELs (i.e. small bowel vs large bowel) should be considered, as different sites may contain different numbers of IELs and phenotypes.

◆◆◆◆◆◆ SEPARATION OF T AND NON-T CELLS FROM MONONUCLEAR CELLS

The E-rosetting Technique

The E-rosetting technique describes a procedure for separating T cells and non-T cells from a population of MNCs (e.g. peripheral blood or synovial fluid MNCs). This method is based on the ability of human T cells to bind to sheep erthrocytes via their CD2 molecule. Neuraminidase treatment of sheep red blood cells (SRBCs) enhances the binding of SRBCs to T lymphocytes (Weiner *et al.*, 1973). First, neuraminidase-treated SRBCs are prepared. Secondly, SRBCs and MNCs are mixed to form rosettes (E^+), which are then isolated from the non-rosetting population (E^-, i.e. B cells and monocytes) by Ficoll gradient centrifugation. In the last step, bound SRBCs are separated from the rosetted T cells by hypotonic lysis (Gmelig-Meyling and Ballieux, 1977).

Equipment and reagents for E-rosetting

- SRBCs (e.g. from Biologische Arbeitsgemeinschaft Hessen, Germany); sterile PBS; suppl. RPMI-1640; FCS, heat inactivated (Life Technologies Inc.); Test-Neuraminidase (Centeon L.L.C., King of Prussia, PA); Ficoll density 1.09 (Biochrom).
- 15-ml conical centrifuge tubes (e.g. Greiner or Falcon); temperature-controlled centrifuge (e.g. Beckman or Heraeus).

Preparation of neuraminidase-treated SRBC

A suspension of SRBCs (2 ml) and sterile PBS (10 ml) are placed in a 15-ml conical centrifuge tube and spun at 2000 rpm (900*g*) for 10 min, whereafter the PBS supernatant is removed and the cells are resuspended in PBS. This washing procedure is repeated twice. Before treatment with neuraminidase, washed SRBCs can be stored at 4°C for 3 days. Part of the dry SRBC pellet (300 µl) is incubated with 4.6 ml RPMI-1640 and 100 µl neuraminidase in a water bath (37°C, 30 min), washed twice with RPMI-1640 (2000 rpm, 10 min), and finally resuspended in RPMI-1640 to a total volume of 5 ml. The suspension is stored at 4°C until use.

Rosette formation and Ficoll density gradient centrifugation

1. MNCs are prepared by standard Ficoll-Hypaque centrifugation (see page 540), washed, counted and suspended in suppl. RPMI-1640/10% HUS (10×10^6 cells ml^{-1}). The neuraminidase-treated SRBCs are mixed with the MNCs (20–30 min, room temperature) to allow E-rosette formation, whereafter the mixture is layered over a Ficoll solution (density 1.09) in a 15-ml conical centrifuge tube. The volumes of SRBCs, medium and Ficoll used in this protocol depend on the number of MNCs to be separated (Table 1). The tubes are centrifuged for 30 min at 2800 rpm.
2. Remove and decant about 80% of the upper layer (RPMI-1640/10% HUS) from the centrifuged suspension. The E-rosette-negative (monocyte/B cell enriched) layer (E^-) is recovered from the interface layer with a pipette, transferred to a 15-ml conical tube, and washed with suppl. RPMI-1640/5% FCS.
3. The E-rosette-positive (T cell) pellet (E^+) is suspended in 1 ml RPMI-1640/10% FCS in the 15-ml tube. Cold distilled water (2 ml) is added for hypotonic lysis of SRBCs and mixed gently. After a few seconds, add 8 ml RPMI-1640/10% FCS. Transfer this suspension to a 50-ml tube containing 40 ml RPMI-1640/10% FCS and centrifuge for 10 min at 1300 rpm.

Pitfalls

After T cells have been exposed to SRBCs some functional activities may be enhanced, as T lymphocytes can be activated via the sheep erythrocyte receptor protein (CD2) to proliferate in an accessory-cell-independent manner (Meuer *et al.*, 1984).

Table 1. Volumes of neuraminidase-treated SRBCs, Ficoll (density 1.09), and RPMI-1640/10% HUS for density gradient centrifugation, depending on the number of MNCs to be separated

MNCs in RPMI-1640/10% HUS cells	No. of MNCs		
	10×10^6 cells in 1.5 ml	$20–30 \times 10^6$ cells in 2 ml	$40–50 \times 10^6$ in 3 ml
Neuraminidase-treated SRBCs	1.5 ml	2 ml	2.5 ml
Ficoll 1.09	2 ml	3 ml	4 ml

◆◆◆◆◆◆ SEPARATION OF T-CELL SUBSETS

Purification of T-cell Populations by Indirect Antibody Panning

T cells expressing particular cell surface markers, such as the CD4, CD8, αβ-TCR or γδ-TCR molecules, can be selected by their capacity to bind to antibody-coated plastic plates (Wysocki and Sato, 1978). For example, to purify $CD8^+$ T cells, isolated T cells (E^+ cells) are treated with a mouse anti-human monoclonal antibody against the CD4 molecule, and then incubated on plastic dishes that have been coated with an anti-mouse immunoglobulin G (IgG) antibody. The T-cell population that is not CD4 positive (i.e. the $\alpha\beta\text{-TCR}^+$ $CD8^+$ and the $\gamma\delta\text{-TCR}^+$ subpopulations), and does not therefore bind the mouse anti-human CD4 antibody, will not adhere to the coated plate. These $CD4^-$ cells can be selected physically from the adherent $CD4^+$ subpopulation.

Equipment and reagents

- T-cell population (E^+ cells).
- Appropriate mouse monoclonal antibody (e.g. OKT4 or OKT8 hybridoma supernatant containing anti-CD4 or anti-CD8 antibodies, or commercially available anti-CD4, anti-CD8 antibody); goat anti-mouse IgG antibody (e.g. Medac, Hamburg, Germany); suppl. RPMI-1640; FCS, heat inactivated; PBS, sterile.
- Plastic, six-well plates (Macroplate Standard, Greiner); 15-ml conical centrifuge tubes (e.g. Falcon); sterile rubber scraper; temperature-controlled centrifuge (e.g. Beckman or Heraeus).

Procedure for the separation of T cells into $CD4^+$ and $CD8^+$ T cells

1. *Preparation of the panning plate.* Goat anti-mouse Ig is diluted to 10 μg ml^{-1} in suppl. RPMI-1640 and added to the wells of a plastic six-well plate (1.5 ml per well). To separate 2×10^6 to 3×10^6 T cells, one well of the panning plate is needed. Incubate overnight at 4°C or for 60 min at room temperature. Remove unbound Ig by using a sterile pipette and gently wash the plate by adding 3 ml PBS to each well (wash three times). Add 2 ml suppl. RPMI-1640/5% FCS and keep the plate at 4°C until the T cells are added to the plate (at least 30 min).
2. *Pretreatment of the T cells.* Prepare the monoclonal antibody (e.g. sterile filtered OKT4 hybridoma supernatant containing these antibodies, or commercially available anti-CD4 antibody diluted in sterile PBS at a concentration appropriate for flow cytometry according to the manufacturer's instructions). Count the T-cell population and

place the cells in a 15-ml centrifuge tube in suppl. RPMI-1640/5% FCS; spin for 10 min at 1300 rpm (400*g*) and 4°C. Decant the supernatant and resuspend the cell pellet in 1–2 ml of the monoclonal antibody hybridoma supernatant or 0.5 ml of the anti-CD4 monoclonal antibody diluted in PBS. Incubate the tube containing the cells for 30 min on ice (iced water) and then fill with suppl. RPMI-1640/10% FCS. Centrifuge for 10 min at 1300 rpm (400*g*) and 4°C. After the supernatant has been removed, the cell pellet is resuspended in suppl. RPMI-1640/10% FCS, and the wash repeated once. Finally, the cells are resuspended in suppl. RPMI-1640 (1.5 ml per 2×10^6 to 3×10^6 cells).

3. *Incubation of the coated plate with the pretreated T cells.* Remove the RPMI-1640/5% FCS from the coated wells of the six-well panning plate with a sterile pipette, and immediately add the pretreated T cells in RPMI-1640 (1.5 ml per well). Spin the plate for 10 min at 300 rpm and 4°C. Carefully remove the plate from the centrifuge, and incubate for another 30 min at 4°C.
4. *Collection of the negatively selected cells.* Gently swirl the plates for 1 min and collect the supernatant containing the non-adherent cells using a sterile pipette. The negatively selected, non-adherent (i.e. $CD4^-$) T cells are washed twice with suppl. RPMI-1640/10% FCS in a 15-ml conical tube, counted, and resuspended in suppl. RPMI-1640/10% human serum or interleukin-2 supplemented medium, depending on the further culture proceedings. This non-adherent population of cells should be 90–95% pure.
5. *Collection of positively selected adherent $CD4^+$ T cells.* Wash the plates gently with 3 ml suppl. RPMI-1640/5% FCS per well (2–3 washes) until all non-adherent cells have been removed (check using an inverted microscope). The adherent T cells are then gently scraped from the wells of the plate using a sterile rubber scraper. This cell population should be approximately 98% pure.

Pitfalls

The purity of the adherent cell population is greater than that of the non-adherent population. However, it must be considered that the function of the adherent T-cell population may be altered by the binding of specific antibodies to surface molecules to be positively selected.

Immunomagnetic Negative Selection of $CD4^+$ T cells

The protocol below is another cell separation technique mediated by antibody–antigen reactions. T cells (E^+ cells) are incubated with specific monoclonal antibodies to surface molecules (anti-CD8) to coat unwanted T cells. Magnetic beads coated with goat anti-mouse IgG are then applied to the cell suspension in order to bind the antibody-coated cells. After binding, the target cells can be recovered using a strong magnetic field. Negative isolation is a method by which the $CD4^+$ subset is purified from the $CD8^+$ subset binding to the coated magnetic beads. Furthermore, in a positive selection step, the beads can be removed from the $CD8^+$ target cells by a process of detachment.

Equipment and reagents

- T-cell population (E^+ cells).
- Appropriate monoclonal antibody (e.g. anti-CD8 antibody by Pharmingen); goat anti-mouse IgG-coated magnetic beads (Dynabeads M-450, Dynal, Oslo, Norway); sterile PBS; FCS, heat inactivated; coating medium (Hanks balanced salt solution (HBSS) without Ca^{2+}, Mg^{2+}, or Phenol Red, supplemented with 10% FCS, 20 mM HEPES); suppl. RPMI-1640; HUS, heat inactivated.
- Magnetic separation device (Dynal MPC-1); mixing device (Dynal MX1, 2 or 3); 15-ml centrifugation tubes (e.g. Falcon); vortex mixer; temperature-controlled centrifuge (e.g. Beckman or Heraeus).

Procedure

(Funderud *et al.*, 1987; *DYNAL Handbook*, 1996.)

All steps in the protocol are done at 4°C.

1. *Prewash of Dynabeads M-450.* Transfer the required number of Dynabeads M-450 from the vial to a polypropylene washing tube containing PBS/2% FCS (washing buffer), and place on the Dynal MPC-1 for 2 min. Decant the supernatant, resuspend in excess washing buffer, and replace on the Dynal MPC-1. Finally, resuspend in a small volume of coating medium (e.g. the volume originally pipetted from the vial).
2. *Antibody coating of $CD8^+$ T cells.* Resuspend washed T cells in 10 ml coating medium at 2×10^7 cells ml^{-1} in a 15-ml conical tube, and add 1 ml anti-CD8 monoclonal antibody at a 10× saturating concentration. Incubate for 30 min at 4°C with gentle tilting and rotation (e.g. in the mixing device).
3. Wash twice in coating buffer (centrifugation at 1000 rpm, 4°C) to remove unbound antibody.
4. Add the suspension of washed Dynabeads and incubate for 30 min at 4°C with gentle tilting and rotation (e.g. in the mixing device) to keep cells and beads in suspension.
5. Place the tube in a Dynal MPC and leave it to rest for 2 min to magnetically remove the $CD8^+$ cells labelled by antibody and coated with beads. Transfer the unbound cells to a fresh tube, perform a second magnetic separation, count, and resuspend in suppl. RPMI-1640/10% HUS. Negatively selected cells obtained by this method are unstimulated, pure and of high yield.
6. For recovery of positively selected $CD8^+$ T cells, remove the tube from the Dynal MPC, and wash the rosetted cells by resuspending in RPMI-1640/10% HUS. Repeat step 5 twice. These positively selected cells can be removed from the beads by a process of detachment (see the notes on the DETACHaBEAD system in the manufacturer's (Dynal) instructions).

◆◆◆◆◆ ESTABLISHMENT OF T-CELL LINES

T-cell lines are cultures of T lymphocytes grown by repeated cycles of stimulation, usually with antigen, antigen-presenting cells and growth factors such as interleukin-2 (IL-2). In the primary reaction, antigen is encountered and processed by the antigen-presenting cells (monocytes, macrophages, B cells). The processed antigen is presented to T lymphocytes, which then secrete a variety of T cell growth factors, primarily IL-2, which are required for the expansion of both $CD4^+$ and $CD8^+$ T cells. For the *in vitro* induction of bacteria-specific T-cell lines, it is important to note that different preparations of the bacterial antigens (e.g. whole viable or killed bacteria, soluble bacterial proteins, usage of bacteria-infected antigen-presenting cells) will selectively or predominantly expand different subsets of T lymphocytes. To generate distinct T-cell ligands, the host cell can process microbial pathogens from two major compartments within the cell. Bacteria that normally proliferate outside the cell may secrete bacterial toxins and soluble proteins, which can be internalized by antigen-presenting cells via endocytosis. These and other bacterial degradation products in the vesicular compartment (endosomes and lysosomes) will deliver peptides that are bound to major histocompatibility complex (MHC) class II molecules. The peptide–MHC class II complex is recognized by $CD4^+$ T cells. When devising experimental strategies for identifying immunologically important proteins of a pathogen, it should be remembered that the native conformation of the bacterial proteins need not be preserved during their isolation, since the T cells are stimulated with linear peptides of proteins complexed with MHC molecules (Shimonkevitz *et al.*, 1983). $CD8^+$ T cells appear to be a critical component of host resistance to intracellular bacteria. Some bacteria (e.g. *Listeria monocytogenes*) replicate in the cytosol or in the contiguous nuclear spaces. Bacterial peptides thus originating in the cytosol are loaded on MHC (HLA) class I molecules in the endoplasmic reticulum. A stable complex of MHC class I molecule, β_2-microglobulin and peptide is expressed on the cell surface, where it is recognized by the antigen-specific receptor (TCR) of $CD8^+$ T cells. Interestingly, even *Salmonella typhimurium*, a facultative intracellular bacterium confined to vacuolar compartments with no active egress into the cytosol, can induce $CD8^+$ T cells responses. This has been shown by Pfeifer *et al.* (1993) who demonstrated an alternative class I processing pathway of *Salmonella* antigens, suggesting that post-Golgi MHC class I molecules might be used for passing antigenic peptides to the cell surface and presenting them to $CD8^+$ CTLs. In human HLA class I-associated disease, we have characterized and propagated HLA-B27 restricted $CD8^+$ T cell lines and clones with specificity for *Yersinia* and *Salmonella* spp. (Hermann *et al.*, 1993). In the latter experiments, bacteria-infected cells can be used as reagents to propagate or screen lymphocytes for responsiveness.

After PBL stimulation with live mycobacteria (Kabelitz *et al.*, 1990; Havlir *et al.*, 1991), or with other live bacteria such as *Salmonella* spp. (Havlir *et al.*, 1991), $\gamma\delta$-TCR^+ cells are found to be the most prominent

population in the resulting T-cell cultures. An enrichment of γδ-TCR$^+$ cells in T-cell lines upon stimulation with live bacteria is even more prominent in synovial lymphocyte cultures as compared with peripheral blood T-cell cultures (Hermann *et al.*, 1992).

It is the purpose of this chapter to describe strategies to stimulate human T cells within a mononuclear cell population, and to obtain T-cell lines with specificity for bacteria or their distinct products. Different protocols will lead to predominant expansion of either αβ-TCR$^+$ CD4$^+$, αβ-TCR$^+$ CD8$^+$, or γδ-TCR$^+$ lymphocytes. In all protocols described here, the culture medium is supplemented with human AB serum instead of FCS, since bovine serum proteins may stimulate human T lymphocytes and therefore induce FCS-specific T-cell lines.

Preparation of Bacteria for *In Vitro* Stimulation of Mononuclear Cells

For *in vitro* stimulation of MNCs it is possible to use either whole bacteria (viable or inactivated) or bacterial fractions. The approach used to generate bacterial preparations will have important implications on the experiment.

We focus here on methods for generating bacterial preparations from *Yersinia enterocolitica* that are straightforward and may be applied to a wide range of experimental situations (Probst *et al.*, 1993a; Duchmann *et al.*, 1995, 1996). In the first two sections, the generation of preparations from whole *Y. enterocolitica* by heat inactivation, sonication or shearing force using beads is described. If available, a French press can be used to disrupt bacterial cells by applying a shearing force. We then give an example of how bacterial cell fractions can be obtained and further purified in order to characterize a clonal T-cell response in more detail, i.e. eventually to define specific antigen(s). When trying to establish your own approach, it may be necessary to consult detailed references (Apicella *et al.*, 1994; Ferro-Luzzi Ames, 1994; Fischetti, 1994; May and Chakrabartry, 1994; Nikaido, 1994; Rosenthal and Dziarski, 1994; Vann and Freese, 1994). Attention should also be paid to the fact that any procedure which includes the use of substances that are toxic to cellular assays needs to be excluded.

Heat-killed bacteria

Equipment and reagents

- Bacterium of interest.
- Appropriate reagents and equipment for culture, e.g. CIN agar (Gibco, Life Technologies Inc.); brain heart infusion (BHI) broth (Merck, Industrial Chemicals, Hawthorne, NY); autoclave; Erlenmeyer flask; spectrophotometer.

Procedures

Y. enterocolitica 03 or 09 are grown on *Yersinia*-selective agar plates (CIN), cultivated in BHI broth for 24 h at 27°C, and harvested at the late exponential phase. Suspend the bacteria in 10 ml PBS and wash twice. Resuspend the washed bacteria in 10 ml PBS and autoclave for 20 min at 121°C. After short vortexing of autoclaved bacterial extract, allow the crude components to sediment. Collect the supernatant and dilute with PBS to an OD (600 nm) of 0.5.

Pitfalls

It has been shown that the cultivation temperature influences the protein expression of *Y. enterocolitica* (Brubaker, 1991) and that heat (1 h, 42°C) induces increased production of 'heat-shock' proteins (Gething and Sambrook, 1992). Heat-killed bacteria may activate $\gamma\delta$-TCR$^+$ rather than $\alpha\beta$-TCR$^+$ CD4$^+$ T cells in some blood donors (Munk *et al.*, 1990), particularly in synovial MNC cultures (Hermann *et al.*, 1992).

Bacterial sonicates

Equipment and reagents

- Bacterium of interest.
- Appropriate reagents and equipment for culture; BHI broth (Merck, Industrial Chemicals); buffer S (HEPES 0.02 M, DNase 0.05 mg ml^{-1} (Sigma), RNase (0.05 mg ml^{-1}) (Sigma), equilibrated as pH 7.5 using 5 N NaOH); sonicator (e.g. Branson Sonifier 250/450 or Labsonic L/U, B. Braun Biotech International); ice; sterile tubes (e.g. Falcon); sterile filter (45 µm).

Procedures:

1. For bacterial culture, incubate 4 ml bacteria in 50 ml BHI and 1.5% agar (Difco) sterilized at 121°C for 15 min, and grow overnight in a bacterial shaker at 37°C. The next day, centrifuge the bacterial cultures at 9000 rpm and wash twice in 0.02 M HEPES buffer (pH 7.5).
2. To disrupt the bacteria, dissolve the bacterial pellet in 5 ml ice-cold buffer S in a 15-ml tube. Surround the tube with ice and sonicate the bacterial suspension four times for 30 s.
3. Sediment non-disrupted cells and cell detritus by centrifugation at 9000 rpm, and filter the supernatant using a 45-µm sterile filter. Determine the protein concentration using the Lowry method (e.g. Bio-Rad protein assay, Bio-Rad Laboratories, Hercules, CA). The use of 50 µg ml^{-1} in lymphocyte assays is a good starting point.

Pitfalls

Sonication is a quick and efficient method for disintegrating cells and avoids the use of reagents that might be toxic in cellular assays. However, in some situations one may find that the procedure is influenced by many variables and therefore difficult to standardize. Factors such as the density of the bacterial suspension (which can be standardized using a spectrophotometer), the type of sonicator, the type of tip used to transduce the oscillations into the buffer, the shape of the tube containing the bacterial suspension, and the temperature during sonication, may influence the completeness of the cellular disintegration process and thus the particle size and antigenicity. Incomplete disintegration of cells and contamination of lymphocyte cell cultures by sonicates that are not sterile can be a problem. Using 4×30 s or 6×20 s may be a good start, but titration of conditions for the individual bacterium and experiment may be necessary to achieve optimal results.

Bacterial disintegration using beads

Equipment and reagents

- Bacterium of interest.
- Appropriate reagents and equipment for culture; buffer S (HEPES 0.02 M, DNase 0.05 mg ml^{-1} (Sigma), RNase (0.05 mg ml^{-1}) (Sigma), equilibrated at pH 7.5 using 5 N NaOH); beads (e.g. 0.1 mm); bead beater (Biospec Products); ice; appropriate tube or vial; sterile filter (45 µm).

Procedures

1. Using an apparatus for disintegration of small amounts of cells (Mini-Bead Beater Model 3110BX, Biospec Products) growth of bacteria as described in step 1 of the preparation of bacterial sonicates will yield more bacteria than needed. Using an apparatus for larger amounts of cells (Biospec Products), the procedure should be upscaled.
2. Using the Mini-Bead beater, fill a 2-ml screw cap vial half to one-third full with beads (0.1 mm). Fill the remaining vial volume with buffer S and bacterial cells (up to 40% wet weight of the solution volume).
3. Using a speed of 5000 rpm the procedure is performed for 6×30 s with intervening cooling on ice.
4. Sediment non-disrupted cells, cell detritus and beads by centrifugation at 9000 rpm, and filter the supernatant using a 45 µm sterile filter. Determine the protein concentration using the Lowry method (Bio-Rad protein assay, Bio-Rad). Use of 50 µg ml^{-1} in the lymphocyte assays may be a good starting point.

Pitfalls

Pitfalls encountered using beads are similar to those that arise when using a sonicator. Variables such as the density of the bacterial cell suspension, the size of the beads, the speed used in centrifugation, the total time of the procedure and the choice of time intervals may be optimized for the individual experiment.

Bacterial cell fractions

This section gives an example of how bacterial cells that have been disintegrated using beads or sonication can be fractionated further. In this protocol, ultracentrifugation results in separation of cytoplasm/periplasm from membranes. The cytoplasm/periplasm fraction is then purified further by precipitation with ammonium sulfate, gel chromatography and reverse-phase HPLC (Probst *et al.*, 1993b). Membranes are purified into outer and inner membranes by saccharose density gradient centrifugation (Hancock and Nikaido, 1978).

Equipment and reagents

- Equipment and reagents for disintegration of cells, as described above.
- HEPES (Biochrom); lysozyme (Sigma); saccharose (Sigma); ammonium sulfate ($(NH_4)_2SO_4$).
- Centrifuge (SW41 and 60 Ti rotor, Beckmann); chromatography unit.

Procedures

1. Disintegrate the bacteria (e.g., *Y. enterocolitica*) using beads or sonication, a described above.
2. Ultracentrifuge the crude bacterial extract (200 000*g*, 60 min, SW41 rotor) and fractionate into cytoplasm/periplasm (supernatant) and membranes (pellet).
3. Fractionate membranes (pellet) into outer and inner membranes by saccharose density-gradient centrifugation. Incubate the pellet containing the membranes in 0.02 M HEPES with 0.05 mg ml^{-1} lysozyme for 30 min, and equilibrate in 11% (v/v) saccharose. Centrifuge a first gradient consisting of 5 ml suspension containing the membranes/11% (v/v) saccarose, 6 ml 15% (v/v) saccharose and 1 ml 70% (v/v) saccharose (39 000 rpm, 1 h, 40°C, SW41 rotor). Overlay the lower 2 ml of the gradient (white-yellow ring) on a second gradient consisting of 1.2 ml 70%, 3 ml 64%, 3 ml 58% and 3 ml 52% saccharose (v/v). Centrifuge this gradient (39 000 rpm, 16 h, 40°C, SW41 rotor) and separate it into four fractions. Wash the total membranes and fractions obtained from the second gradient twice in 0.02 M HEPES pH 7.5 (41 000 rpm, 1 h, 40°C, 60 Ti rotor). Resuspend the pellets in 1–2 ml 0.02 M HEPES pH 7.5, sterile filter and store frozen.

4. Further fractionate cytoplasm/periplasm (supernatant) by sequential ammonium sulfate precipitation using saturations of 0–26%, 26–38%, 38–51%, 51–54% and 64–78% ammonium sulfate. The different concentrations of ammonium sulfate are prepared using saturated $(NH_4)_2SO_4$ solution in 0.05 M Tris/HCl pH 7.5. Precipitate the proteins for 15 min at 40°C while stirring. After centrifugation (10 000*g*, 20 min, 40°C), resuspend the pellet in 0.05 M Tris/HCl pH 7.5 and desalt using Sephadex G25 columns (Pharmacia).
5. For further biochemical fractionation, desalted proteins can be subjected to gel chromatography (e.g. using Sephacryl S300 Superfine columns, Pharmacia), anion exchange chromatography (e.g. using Mono Q columns, Pharmacia) or subjected to reverse-phase HPLC.

In Vitro Generation and Restimulation of Antigen-specific T-cell Lines

Protocol for predominant expansion of CD4$^+$ (and γδ-TCR$^+$) bacteria-specific T-cell lines

Equipment and reagents

- MNC population isolated by Ficoll-Hypaque density centrifugation from heparinized peripheral blood, synovial fluid, synovial membrane, gut mucosa or another source.
- Suppl. RPMI-1640; human AB serum (HUS, heat inactivated); recombinant IL-2 (rIL-2, e.g. Boehringer Mannheim, Indianapolis, IN); bacterial antigen preparation of interest (heat-killed bacteria, bacterial sonicates, or bacterial cell fractions, see page 553–556).
- Sterile 24-well flat-bottomed microtitre plates (e.g. Costar, Cambridge, USA); 15-ml conical centrifuge tubes (e.g. Greiner or Falcon); humidified 37°C, 5% CO_2 incubator (e.g. Heraeus); ^{137}Cs source for irradiation.

Note: When using whole heat-killed bacteria or viable bacteria (which will be killed by antibiotics after a short incubation with the MNCs), γδ-TCR$^+$ rather then αβ-TCR$^+$ CD4$^+$ cells may be stimulated and expanded in some donors.

Procedure

(Probst *et al.*, 1993a.)

1. *Antigen-specific stimulation - I.* Resuspend unfractionated, washed MNCs in suppl. RPMI-1640/10% HUS (1.5×10^6 cells ml^{-1} of culture medium). Place 3×10^6 MNCs in the wells of a 24-well microtitre plate at a volume of 2 ml suppl. RPMI-1640/10% HUS and add the soluble

bacterial antigen at an optimal concentration. (This optimal concentration (usually 5–50 μg ml^{-1} protein) should have been determined before starting the generation of T-cell lines, using a standard lymphocyte proliferation assay.) Incubate plates for 8 days in a humidified 37°C, 5% CO_2 incubator. After 3 days of culture, gently pipette off the medium fluid from the wells. Replace the medium by rIL-2 (20 U ml^{-1}) supplemented RPMI/10% HUS (without antigen). Inspect wells for growing cells microscopically and for the colour of the medium every 2 days. If necessary (colour changing to yellow), split the cultures using fresh rIL-2 (20 U ml^{-1}) supplemented RPMI/10% HUS culture medium.

2. *Antigen-specific restimulation - II.* Restimulate the resulting T-cell line with antigen and with autologous irradiated peripheral blood MNCs as feeder cells. (To obtain the feeder cells, prepare blood MNCs from the same T-cell donor by Ficoll-Hypaque density centrifugation, as described above.) Count the washed MNCs, irradiate (4000 rad from a ^{137}Cs source), suspend in RPMI-1640/10% HUS/20 U ml^{-1} rIL-2 (2×10^6 to 3×10^6 cells ml^{-1}), and keep on ice until needed. (For ease of manipulation without having to draw blood several times from the same donor, larger numbers of blood MNCs can be irradiated, aliquoted, cryopreserved and recovered as needed.) Pool cells from all wells of the 24-well microtitre plate in 15-ml conical centrifuge tubes. Fill the tubes with suppl. RPMI-1640/5% HUS and centrifuge for 10 min at 1300 rpm (400*g*) and 18°C. After removing the supernatant, resuspend the cell pellet in suppl. RPMI-1640/10% HUS/20 U ml^{-1} r-IL2 at 1×10^6 cells ml^{-1}. Pipette 1 ml into each well of a 24-well microtitre plate and co-incubate these responder T cells with 2×10^6 to 3×10^6 irradiated autologous feeder cells at a total volume of 2 ml. Add the optimal concentration of the bacterial antigen preparation and incubate the plates in a humidified 37°C, 5% CO_2 incubator. Feed the cultures with suppl. RPMI-1640/10% HUS/20 U ml^{-1} r-IL2 medium, or split the cultures if necessary.
3. After 8 days, wash the T-cell line and restimulate it as described in step 2. By checking the wells microscopically, one may detect many dead cells or detritus. The detritus can be removed by purifying viable T cells by Ficoll-Hypaque density gradient centrifugation. Co-incubate the T cells with autologous feeder cells and antigen for an additional 8 days.
4. At this point (day 24 of T-cell culture), analysis of phenotypes (e.g. FACS analysis) and antigen specificity (e.g. lymphocyte proliferation assay) of the T-cell line can be done. Furthermore, T-cell clones can be prepared from the T-cell line (see page 561).

Protocol for bacteria-specific MHC class I restricted $CD8^+$ specific T cells

Certain human rheumatic diseases, namely the so-called spondylarthropathies, are strongly associated with the MHC class I molecule HLA-B27 and with antecedent infection with Gram-negative bacteria. The latter is proven in *Yersinia, Shigella, Salmonella* and *Chlamydia* induced reactive

arthritis, but is also strongly implicated in ankylosing spondylitis. The function of HLA-B27 in the presentation of bacterial and self-peptides is considered to be crucial in the pathogenesis of the spondylarthropathies. This theory has been supported by the identification of synovial fluid cytotoxic T lymphocyte (CTL) clones that specifically killed *Yersinia* or *Salmonella* infected HLA-B27$^+$ target cells (Hermann et al., 1993). It is known, however, that the stimulation of synovial fluid MNCs with soluble bacterial antigens produces CD4$^+$ T-cell lines, and that stimulation with live bacteria leads to an enrichment of TCR-$\gamma\delta^+$ cells (up to 90% of all CD3$^+$ T cells), whereas bacteria-specific MHC class I restricted cytotoxicity is not detectable in these T-cell cultures (Hermann et al., 1992). Therefore, to generate MHC class I-restricted *Yersinia* or *Salmonella* responsive CTL lines, one has to apply culture conditions that are specific to the facultatively intracellular nature of the bacteria, and the antigen should be presented optimally for class I, that is endogenously, for example by infection of human MHC class I transfected cell lines with live bacteria. We describe here the generation of HLA-B27 restricted CTL lines with specificity for *Y. enterocolitica* as an example of the propagation of MHC class I-restricted bacteria-specific CTL.

Equipment and reagents

- Synovial fluid MNCs derived from an inflamed joint of a patient (HLA-B27$^+$) with *Yersinia*-induced reactive arthritis (for the isolation of synovial fluid MNCs by Ficoll-Hypaque density gradient centrifugation, see page 540).
- HLA-B*2705- and human β_2-microglobulin-transfected mouse L-cells (B27-L-cells) (described by Kapasi and Inman, 1992).
- A patient isolate of *Y. enterocolitica* O3 or O9, grown for 24 h at 27°C in BHI broth (Merck, Industrial Chemicals).
- PBS; DMEM (Gibco, Life Technologies Inc.) supplemented with 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin (Pen-Strep, Biochrom), and HAT supplement (Gibco Life Technologies Inc.); RPMI-1640 medium without antibiotics (Gibco Life Technologies Inc.); gentamycin (Merck, Industrial Chemicals); suppl. RPMI-1640 (see page 540); FCS, heat inactivated; human AB serum (HUS), heat inactivated; rIL-2, Boehringer Mannheim); Ficoll-Hypaque (Histopaque-1077, Sigma).
- 15-ml conical centrifuge tubes (e.g. Greiner); sterile 12-well flat-bottomed plates (Costar); sterile 96-well round-bottomed microtitre plates (e.g. Nunc); sterile rubber scraper; temperature-controlled centrifuge with GH-3.7 horizontal rotor (e.g. Heraeus or Beckman); humidified 37°C, 5% CO_2 incubator (e.g. Heraeus).

Procedures

1. *Growth and infection of stimulator cell line* (Hermann *et al.*, 1993; Huppertz and Heesemann, 1996). Grow HLA-B*2705- and human

β_2-microglobulin-transfected mouse L-cells (B27-L-cells) in 12-well plates in DMEM containing antibiotics (penicillin/streptomycin), 10% FCS and HAT supplement. Cultivate *Y. enterocolitica* in BHI broth for 24 h at 27°C, and harvest at the late exponential phase. Wash a bacterial suspension and resuspend in PBS, diluted to an OD (600 nm) of 0.1. Thereafter dilute 1 : 20 in RPMI-1640 without antibiotics (at a multiplicity of infection of 50 yersiniae per L cell, or 5×10^6 yersiniae per 10^5 L cells). Wash subconfluent adherent monolayers of B27-L cells with RPMI-1640 (without antibiotics). For B27-L cell infection, add 2 ml of the suspension of live *Y. enterocolitica* in RPMI-1640 (without antibiotics) to each well of the 12-well plate, and incubate at 37°C, 5% CO_2 in a humidified atmosphere. After 2 h of incubation, add gentamycin 100 µg ml^{-1} to the cultures to kill extracellular bacteria. After an additional 1 h of culture, carefully remove the supernatant from the adherent cells by using a sterile pipette. Gently wash the wells three times by adding and removing 3 ml RPMI-1640.

2. *Stimulation of synovial fluid MNCs* (Hermann *et al.*, 1993). The *Yersinia*-infected B27-L cells are used as stimulators for synovial fluid MNCs derived from a HLA-B27$^+$ patient. Add 2×10^6 MNCs in 2 ml suppl. RPMI-1640/10% HUS to each of the wells containing *Yersinia*-infected B27-L cells, and incubate in a humidified 37°C, 5% CO_2 incubator. After 24 h of co-culture, L-cell detritus must be removed from the human responder cells. The supernatant containing the non-adherent human cells, killed bacteria and cell detritus is pooled from all wells of the 12-well plate in 15-ml conical centrifuge tubes. In addition, gently scrape the remaining adherent cells (i.e. L cells with attached human cells) from the wells using a sterile rubber scraper. Pool the cells and centrifuge the suspensions through a Ficoll-Hypaque density gradient to recover the viable human cells at the interface, and wash twice in suppl. RPMI-1640/5% FCS. Resuspend the cell pellet in suppl. RPMI-1640/10% HUS/20 U ml^{-1} rIL-2 at 1×10^6 cells ml^{-1}. Pipette 100 µl cell suspension into the wells of a 96-well round-bottomed microtitre plate and incubate for 8 days. If necessary, feed the cultures with suppl. RPMI-1640/10% HUS/20 U ml^{-1} rIL-2 medium, or split the cultures.
3. *Restimulation of the T-cell line.* Prepare *Yersinia*-infected B27-L cells as described in step 1. Pool the cells from all wells of the 96-well plate into 15-ml conical centrifuge tubes, and fill the tubes with suppl. RPMI-1640/5% HUS. Centrifuge for 10 min at 1300 rpm (400*g*) and 18°C. After removing the supernatant, resuspend the cell pellet in suppl. RPMI-1640/10% HUS/20 U ml^{-1} rIL-2 at 1×10^6 cells ml^{-1}. Add 2 ml of this cell suspension to each of the wells containing *Yersinia*-infected B27-L cells and incubate in a humidified 37°C, 5% CO_2 incubator. After 24 h of co-culture, purify the T-cell lines by Ficoll-Hypaque density gradient centrifugation, and wash and seed them into the wells of a 96-well round-bottomed plate, as described in step 2.

4. After another 8 days of culture, $CD8^+$ T cells are negatively selected from the T-cell line by indirect antibody panning (see page 549). These $CD8^+$ T cells can now be cloned using a non-specific cloning protocol (see page 565); an antigen-specific protocol is not useful in this instance.

◆◆◆◆◆◆ ESTABLISHMENT OF T-CELL CLONES

A T-cell clone is a continuously growing line of T cells derived from a single progenitor cell. The generation of T-cell clones helps in the identification of even minor fractions of T-cell populations and the investigation of effector functions that are stable within each line. Similar to T cell lines, T-cell clone cells must be stimulated periodically with antigen and antigen-presenting cells in order to maintain growth. However, if the antigen is not yet known or not available in sufficient quantities, non-specific methods of restimulation of T-cell clones can be used (see page 565).

Representative Cloning Protocol

This protocol provides direct cloning of T cells (from unseparated MNCs) without prior antigen-specific *in vitro* stimulation or selection for certain T-cell phenotypes (Fleischer and Bogdahn, 1983; Hermann *et al.*, 1989).

Equipment and reagents

- MNCs to be cloned (from peripheral blood or other sources), isolated by Ficoll-Hypaque density gradient centrifugation (as described on page 540), or purified T-cell population (E^+ cells, see page 547–548).
- Allogeneic blood MNC (feeder cells), isolated by Ficoll-Hypaque density gradient centrifugation from a buffy coat.
- Suppl. RPMI-1640; human AB serum (HUS), heat inactivated; rIL-2 (Boehringer Mannheim, Mannheim, Germany); phytohaemagglutinin (PHA-P) (e.g. Biochrom).
- A 0.45 µm sterile filter; plastic flasks; 15-ml conical centrifuge tubes (e.g. Greiner or Falcon); Terasaki microtest plates (Nunc, Roskilde, Denmark); 96-well round-bottomed microtitre plates (Nunc); 24-well flat-bottomed plates (e.g. Costar, Cambridge, USA); humidified 37°C, 5% CO_2 incubator (e.g. Heraeus); ^{137}Cs source for irradiation.

Procedure

1. *Preparation of conditioned medium (T-cell growth factor).* T-cell growth factor (TCGF) is generated from PHA-P activated allogeneic blood MNCs. Suspend MNCs at 1×10^6 cells ml^{-1} in suppl. RPMI-1640/2%

HUS containing 1 μg ml^{-1} PHA-P and incubate the cells in plastic flasks in a humidified 37°C, 5% CO_2 incubator, each flask containing 200 ml cell suspension. After 24 h, collect the supernatant and pass it through a 0.45-μm filter.

2. *Preparation of feeder cells.* Irradiate allogeneic peripheral blood MNCs (feeder cells) (4000 rad from a ^{137}Cs source) and resuspend in TCGF at a cell concentration of 1×10^6 cells ml^{-1}. To this cell suspension add 1 μg ml^{-1} PHA-P. Plate 10 μl of the cell suspension into each well of 60-well Terasaki plates. (We recommend that at least 25 plates (i.e. 1500 wells) are prepared in order to achieve a sufficient number of growing T-cell clones.)
3. *Preparation and seeding of cells to be cloned.* Count the MNCs (T cells) to be cloned several times, resuspend in suppl. RPMI-1640/10% HUS/20 U ml^{-1} rIL-2 at 5×10^2 cells ml^{-1} (= 5 cells per 10 μl) and mix vigorously before plating at the limiting dilution in the prepared Terasaki plates. (The limiting dilution can be determined by seeding the T cells at 5, 1, and 0.5 cells per well in 10 μl medium.)
4. *Incubation.* Incubate the plates for 8–12 days in a humidified 37°C, 5% CO_2 incubator. Inspect the wells for growing clonal T-cell colonies microscopically.
5. Transfer the growing T-lymphocyte clones (TLCs) to larger plates (96-well microtitre plates) and restimulate. Prepare irradiated allogeneic feeder cells at 1×10^6 cells ml^{-1} TCGF/1 μg ml^{-1} PHA-P and pipette 100 μl into the wells of 96-well microtitre plates (1×10^5 cells per well). Each growing TLC is transferred to one of the prepared wells. During the following days of incubation, it is important to inspect the wells microscopically every 2 days in order to feed or split the colonies with culture medium (suppl. RPMI-1640/10% HUS/20 U ml^{-1} rIL-2) if necessary, or to transfer them to 24-well plates with additional culture medium.
6. After 10 days of culture, test clones for antigen specificity or phenotype (see page 567).
7. TLCs of interest are propagated further by restimulation with rIL-2 and oxidized stimulator cells (see page 566).

Establishment of T-cell Clones from Bacteria-specific T-cell Lines

Equipment and reagents

- Antigen-specific T-cell line to be cloned (generated as described on page 557). Autologous blood MNCs (feeder cells), isolated by Ficoll-Hypaque density gradient centrifugation from heparinized peripheral blood.
- Suppl. RPMI-1640; bacterial antigen at an optimal concentration; HUS, heat inactivated; rIL-2 (Boehringer Mannheim Corporation).

- 15-ml conical centrifuge tubes (e.g. Greiner or Falcon); 96-well round-bottomed microtitre plates (Nunc); 24-well flat-bottomed plates (Costar); humidified 37°C, 5% CO_2 incubator (e.g. Heraeus); ^{137}Cs source for irradiation.

Procedure

1. *Preparation of feeder cells.* Irradiate (4000 rad from a ^{137}Cs source) autologous peripheral blood MNCs (feeder cells) and resuspend them in suppl. RPMI-1640/10% HUS/20 U ml^{-1} rIL-2 at a cell concentration of 1×10^6 cells ml^{-1}. Add the bacterial antigen preparation at optimal concentration and pipette 100 µl cell suspension into the wells of 96-well microtitre plates (1×10^5 cells per well).
2. *Preparation and seeding of cells to be cloned.* Count the T cells to be cloned several times, resuspend them in suppl. RPMI-1640/10% HUS/20 U ml^{-1} rIL-2 at 1×10^2 cells per ml (i.e. 5 cells per 50 µl), add the bacterial antigen preparation at optimal concentration, and mix vigorously before plating them at limiting dilution in the prepared 96-well plates. (The limiting dilution can be determined by seeding the T cells at 5, 1 and 0.5 cells per well in 50 µl culture medium.)
3. *Incubation.* Incubate plates for 8 days in a humidified 37°C, 5% CO_2 incubator. After that time, inspect wells for growing clonal T-cell colonies microscopically. Between days 8 and 12 of culture, growing TLCs can be fed with medium (suppl. RPMI-1640/10% HUS/20 U ml^{-1} rIL-2) or split in a new 96-well microtitre plate.
4. Growing TLCs are then transferred to larger (24-well) plates and specifically restimulated with irradiated autologous MNCs and antigen (see page 564). Split and transfer to 12-well plates, if necessary.
5. After another 10 days of culture, test clones for antigen specificity and/or phenotype (see page 567).
6. TLCs of interest are propagated further by non-specific restimulation with rIL-2 and oxidized stimulator cells (see page 566).

Specific and Non-specific Restimulation of T-lymphocyte Clones

Long-term growth of human TLCs with proliferative activity is not possible in IL-2 containing medium alone, since with increasing time after stimulation of the TLCs the expression of activation antigens such as IL-2 receptor and HLA-DR antigens decreases, and the cells lose their blastoid appearance and return to a resting state (Fleischer, 1988). Further periodical stimulation with specific antigen and autologous antigen-presenting cells is then necessary in order to obtain IL-2 receptor expression and a response to IL-2, and thus to maintain long-term growth *in vitro*. Alternatively, methods for a non-antigen-specific stimulation of antigen-dependent proliferative or cytotoxic human TLCs can be applied.

Specific restimulation of T-lymphocyte clones

In cases in which the specificity of the TLCs to be propagated is known and the appropriate antigen is available (e.g. crude preparations of heat-killed or sonicated bacteria, or defined bacterial antigens), periodic stimulation of the TLCs is achieved by recognition of antigen and MHC-encoded molecules. Autologous irradiated PBLs are used as antigen-presenting cells. If HLA restriction molecules of antigen recognition are known, HLA-matched allogeneic PBLs can be used as well. TLCs are restimulated in intervals of 7–9 days.

Equipment and reagents: preparation of autologous antigen presenting cells and for antigen-specific stimulation of TLCs

- Heparinized autologous peripheral blood; cloned T cells to be restimulated.
- Ficoll-Hypaque (Histopaque-1077, Sigma), suppl. RPMI-1640; FCS, heat-inactivated; HUS, heat-inactivated; rIL-2 (Boehringer Mannheim Corporation).
- 50-ml conical centrifuge tubes (e.g. Greiner); 15-ml conical centrifuge tubes (e.g. Falcon); 24- and 12-well flat-bottomed plates (Costar); a ^{137}Cs source for irradiation; temperature-controlled centrifuge; humidified 37°C, 5% CO_2 incubator (e.g. Heraeus).

Procedure

MNCs are obtained from heparinized autologous whole blood by Ficoll-Hypaque density gradient centrifugation and washed as described on page 540. The cells are counted, resuspended in suppl. RPMI-1640/10% HUS/20 U ml^{-1} rIL-2 (1×10^7 cells ml^{-1} in a 15-ml conical centrifuge tube) and kept on ice. Before use as stimulator cells, the PBLs are irradiated (4000 rad from a ^{137}Cs source). The cloned T cells are washed with suppl. RPMI-1640/5% FCS, counted and resuspended in IL-2 medium (1×10^6 cells ml^{-1}). These TLCs (1×10^6) are incubated with 4×10^6 irradiated autologous or HLA-matched antigen-presenting cells in 3 ml IL-2 medium in 12-well plates. For smaller numbers of cloned T cells, 0.5×10^6 T cells are incubated with 2×10^6 to 3×10^6 irradiated antigen-presenting cells in 2 ml suppl. RPMI-1640/10% HUS/20 U ml^{-1} rIL-2 medium in 24-well plates. The appropriate bacterial antigen is added in an optimal concentration. (The optimal concentration is determined in a standard lymphocyte proliferation assay using antigen concentrations of 1–100 µg ml^{-1} in suppl. RPMI-1640/10% HUS.) The plates are cultured in an incubator at 37°C, 5% CO_2 in a humidified atmosphere. IL-2 medium is added to the cultures every 2–3 days, and growing cultures are split with IL-2 medium if necessary.

Pitfalls

It has been reported that $CD4^+$ TLCs may lose their proliferative function when cultivated for longer than 30–35 population doublings (Pawelec *et al.*, 1986). The growth rate usually slows down after 8–10 weeks if stimulated with antigen and autologous PBLs alone. In many cases, such clones can be readily expanded using the non-specific stimulation protocol with oxidized stimulator cells (see page 566).

Non-specific propagation of T-lymphocyte clones

In many cases, such as during the investigation of functional properties of T cells cloned from biological fluids, the specificities of the T lymphocytes to be propagated are not known. Another major limitation of successful long-term culture of human antigen-specific, HLA-restricted TLCs can be the supply of sufficient autologous or HLA-typed cells for use as antigen-presenting cells. Therefore, alternative methods of TLC restimulation are required. In this section we describe the method of non-specific propagation of $\alpha\beta$-TCR^+CD4^+, $\alpha\beta$-TCR^+CD8^+ and $\gamma\delta$-TCR^+ TLCs by mitogenic lectins (phytohaemagglutinin or concanavalin A), by anti-CD3 mAb, or by oxidized stimulator cells. For these methods, irradiated allogeneic peripheral blood MNCs are required as stimulator cells.

Equipment and reagents: preparation of allogeneic stimulator cells

- One buffy coat; Ficoll-Hypaque (Histopaque-1077, Sigma); RPMI-1640; FCS, heat inactivated (Life Technologies Inc.);
- 50-ml conical centrifuge tubes (e.g. Greiner); 15-ml conical centrifuge tubes (e.g. Falcon); a ^{137}Cs source for irradiation; temperature-controlled centrifuge; humidified 37°C, 5% CO_2 incubator (e.g. Heraeus).

Procedure

MNCs are obtained from buffy coats by Ficoll-Hypaque density gradient centrifugation and washed as described on page 540. Washed MNCs are counted, irradiated (4000 rad from a ^{137}Cs source), suspended in RPMI-1640/10% HUS/20 U ml^{-1} rIL-2 (2×10^6 to 3×10^6 cells ml^{-1}), and kept on ice until needed.

Non-specific stimulation of T-lymphocyte clones by mitogenic lectins or anti-CD3 mAb

Mitogenic lectins such as phytohaemagglutinin (PHA) or concanavalin A (ConA) as well as the mitogenic monoclonal antibody anti-CD3 can be used to stimulate TLCs in the presence of accessory cells.

Equipment and reagents

- Irradiated allogeneic MNCs (prepared as described above).
- suppl. RPMI-1640, PHA-P (Biochrom); ConA (e.g. Biochrom); anti-CD3 monoclonal antibody (e.g. Pharmingen); human AB-serum, heat-inactivated; FCS, heat-inactivated.
- 12-well flat-bottomed plates (Costar, Cambridge, MA); 15-ml conical centrifuge tubes (e.g. Falcon); humidified 37°C, 5% CO_2 incubator (e.g. Heraeus).

Procedure

Irradiated stimulator cells (3×10^6) are incubated with 5×10^5 cloned T cells in suppl. RPMI-1640 in the presence of either PHA-P (1 μg ml^{-1}) or ConA (1 μg ml^{-1}) or anti-CD3 (0.1 μg ml^{-1}). Use HUS as medium supplement with lectins, and FCS with anti-CD3.

Pitfalls

The use of lectins or anti-CD3 to obtain long-term growth of TLCs may have disadvantages (Fleischer, 1988): (1) lectin accumulation on the surface of the responder T cells may have toxic effects; (2) lectin or anti-CD3 antibody can lead to mutual killing if the clones possess cytotoxic activity; and (3) lectin or anti-CD3 antibody bound on the cell surface can be carried into experimental assay systems and have disruptive effects in functional tests. In some cases, loss of function of restimulated TLCs can be traced back to mycoplasma contamination of the T-cell clones. The risk of mycoplasma infection is especially high if tumour cell lines grown in FCS are incorporated into the stimulation cultures of TLCs or if FCS has to be used for stimulation, e.g. with the anti-CD3 monoclonal antibody (Padula *et al.*, 1985). In case of mycoplasma infection, the cell lines can be treated with antibiotics such as BM-Cyclin (Boehringer Mannheim Corporation) according to the manufacturer's instructions.

Non-specific propagation of T-lymphocyte clones by oxidized stimulator cells

This method (Fleischer, 1988) is based on the selective modification of the stimulator cells by oxidation of surface galactose residues to reactive aldehydes. The stimulation of responder T cells in this system is due to a covalent cross-linking of stimulator and responder cells via Schiff bases (Fleischer, 1983). The oxidizing enzyme galactose oxidase is removed before adding the responding T cells and, therefore, has no disruptive effects in later assay systems.

Equipment and reagents

- Irradiated allogeneic MNCs (prepared as described on page 540).
- RPMI-1640; test neuraminidase (Centeon L.L.C.), galactose oxidase (Sigma); galactose (Sigma).
- 15-ml conical centrifuge tubes (e.g. Falcon); 12-well flat-bottomed plates (e.g. Costar); temperature-controlled centrifuge; humidified 37°C, 5% CO_2 incubator (e.g. Heraeus).

Procedure

Irradiated (4000 rad) allogeneic MNCs ($4 \times 10^7 ml^{-1}$) are incubated with 0.02 U ml^{-1} neuraminidase and 0.05 U ml^{-1} galactose oxidase in serum-free RPMI-1640 for 90 min at 37°C (15-ml conical tubes). The cells are washed three times in suppl. RPMI-1640 containing 0.01 M galactose to remove residual galactose oxidase bound to galactose residues on the cells. After the last wash, resuspend the cell pellet in suppl. RPMI-1640/10% HUS, rIL-2 20 U ml^{-1}. Oxidized stimulator cells (3×10^6) are then incubated with 5×10^5 cloned T cells in 12-well plates at a total volume of 2 ml. Incubate the plates for 8 days, feed or split the cultures with suppl. RPMI-1640/10% HUS, rIL-2 20 U ml^{-1} medium, if necessary.

Phenotypic Analysis of High Numbers of T-cell Clones

FACS analysis according to standard procedures with mouse anti-human monoclonal antibodies (e.g. anti-CD3, anti-αβ-TCR, anti-γδ-TCR, anti-CD4, anti-CD8) and FITC labelled second antibodies (goat anti-mouse immunoglobulin) is often used to phenotypically characterize T-cell clones and to identify certain T-cell subsets of interest. When high numbers (> 100) of T-cell clones are produced by the method described above, and when only a limited number of cloned T cells is available for analysis, we recommend an enzyme-linked immunoassay (ELISA) for the detection of surface antigens on individual cells (Holzmann and Johnson, 1983), which has a sensitivity comparable to immunofluorescence and can be readily used for the investigation of T-cell populations.

Equipment and reagents

- Cloned T cells suspended in PBS (approximately 1.5×10^4 cells in 100 µl).
- Poly-L-lysine (Sigma); glutaraldehyde (Merck, Industrial Chemicals); monoclonal antibodies (anti-CD3, anti-αβ-TCR, anti-γδ-TCR, anti-CD4, anti-CD8) either as undiluted antibody containing supernatant from hybridomas or as antibody dilutions from purchased reagents prepared in PBS/10% FCS; peroxidase coupled rabbit anti-mouse Ig (e.g. Dakopatts, Hamburg, Germany); 3-amino-

9-ethylcarbazol (Sigma); 0.002% H_2O_2; 0.05 M Tris-HCl pH 7.6; dimethylsulfoxide (DMSO) (Merck, Industrial Chemicals); storage buffer (PBS containing 60% glycerine (Sigma) and 0.1% NaN_3 (Sigma).

- Terasaki microtest plates (e.g. Nunc).

Procedure

Terasaki microtest plates (one plate per monoclonal antibody tested) are treated with poly-L-lysine by flooding the plate with 10 ml PBS containing 25 μg ml^{-1} poly-L-lysine and then washed with PBS (Holzmann and Johnson, 1983). All washing steps are performed by dipping and flicking, whereafter the plates are carefully blotted dry with a paper towel to avoid subsequent dilution of reagents. From each T-cell clone to be investigated, 10 μl of cells (approximately 1500 cells per well) are added to the wells of the Terasaki plates. The plates are centrifuged for 5 min at 90*g* (4°C) to produce an even monolayer of attaching cells, and fixed for 5 min at room temperature by flooding the plate with 10 ml 0.20% glutaraldehyde in PBS. After three washes, 10 ml suppl. RPMI-1640/10% FCS is added for 1 h (37°C, 5% CO_2) to block any remaining protein binding sites. After another six washes, 10 μl undiluted monoclonal antibody-containing hybridoma supernatant or monoclonal antibody dilutions from purchased reagents prepared in PBS/10% FCS is added to the wells for 1 h at 4°C, after which the plates are washed 10 times.

A 1 : 50 dilution (total volume 10 μl) of peroxidase coupled goat anti-mouse Ig (in PBS/10% FCS) is added to each well for 15 min at room temperature. The plates are washed 10 times and then flooded with 10 ml of substrate solution consisting of 0.07 mg ml^{-1} 3-amino-9-ethylcarbazol, 0.002% H_2O_2 in 0.05 M Tris-HCl, pH 7.6. (The carbazol must initially be dissolved in 1/30 volume of DMSO). It is important to note that the substrate solution should be freshly prepared (e.g. during the incubation time of the peroxidase coupled goat anti-mouse Ig). The cells are allowed to react for 30–45 min and the plates are then washed three times in PBS and flooded with storage buffer (PBS containing 60% glycerine, 0.1% NaN_3). Positive-staining cells can be detected by examining the wells using an inverted microscope.

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3 Growth Transformation of Human T Cells

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INTRODUCTION

Molecular and biochemical studies of human T lymphocytes are limited for mainly two reasons. First, T lymphoblastic tumour lines such as Jurkat (Schneider *et al.*, 1977) have a strongly altered phenotype in comparison to primary cells with respect to signal transduction (Bröker *et al.*, 1993) and gene regulation. Secondly, primary T-cell cultures are limited in their lifespan. It is laborious and frequently impossible to grow primary T lymphocytes to large cell numbers, and it requires considerable effort to amplify the T cells in periodic response to a specific antigen and accessory cells expressing the appropriate human leukocyte antigen (HLA) restriction elements. Moreover, impurities due to the addition of irradiated feeder cells may cause difficulties in interpretation of results. The immortalization of human T cells should be the ideal way to solve such problems. Various approaches have been applied for this purpose. The technique of T cell fusion hybridomas has been successful in the murine system. However, this method proved to be much more difficult with human T cells and to be hampered by genomic instability of the clones. Virus-mediated transformation is an established routine method for human B cells, which are efficiently immortalized by Epstein–Barr virus (EBV) (Nilsson and Klein, 1982; Tosato, 1997). EBV-transformed lymphoblastoid B cells retain their antigen-presenting capability and are widely used to study T-cell antigen specificity. Human T cell leukaemia

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virus type 1 (HTLV-1) offers a solution to the problem for human T lymphocytes: HTLV-1 transforms human T cells to stable growth in culture (Miyoshi *et al.*, 1981; Yamamoto *et al.*, 1982; Yoshida *et al.*, 1982; Popovic *et al.*, 1983; Faller *et al.*, 1988; Persaud *et al.*, 1995). For many applications, this approach proved useful, although the transformation is largely confined to $CD4^+$ T cells. Retrovirus-transformed T lymphocytes produce HTLV-1 virions regularly. Unfortunately, the cells tend to lose their T-cell receptor (TcR) complex, their cytotoxic activity and their dependence on interleukin-2 (IL-2) after prolonged cultivation (Inatsuki *et al.*, 1989; Yssel *et al.*, 1989). In this chapter we summarize the procedures for viral transformation of human lymphocytes. The main emphasis is on using cell-free herpesvirus saimiri C488 for the targeted transformation of human T lymphocytes to antigen-independent growth and to an activated phenotype (Biesinger *et al.*, 1992).

Herpesvirus saimiri (Meléndez *et al.*, 1968) is the prototype of the genus γ_2-herpesviruses or rhadinoviruses. This virus is termed 'saimirine herpesvirus type 2' (SHV-2) by the International Committee for the Taxonomy of Viruses (Roizman *et al.*, 1992). However, this term occurs only rarely in the literature. 'H. saimiri' is frequently used as abbreviation for herpesvirus saimiri, while 'HVS' is often mixed up with 'HSV' (herpes simplex virus). The closest relative of herpesvirus saimiri in humans is human herpesvirus type 8, which is associated with Kaposi's sarcoma (Chang *et al.*, 1994). Herpesvirus saimiri is not pathogenic in its natural host, the squirrel monkey (*Saimiri sciureus*), and can easily be isolated from the peripheral blood of most individuals (Falk *et al.*, 1972; Wright *et al.*, 1976). In other South American monkeys such as common marmosets (*Callithrix jacchus*), cotton top marmosets (*Saguinus oedipus*) and owl monkeys (*Aotus trivirgatus*), as well as in certain rabbits (Ablashi *et al.*, 1985; Medveczky *et al.*, 1989), herpesvirus saimiri causes fulminant polyclonal T-cell lymphomas and acute lymphatic leukaemias (reviewed in Fleckenstein and Desrosiers, 1982; Trimble and Desrosiers, 1991; Biesinger and Fleckenstein, 1992). Furthermore, marmoset T lymphocytes can be transformed *in vitro* by herpesvirus saimiri (Schirm *et al.*, 1984; Desrosiers *et al.*, 1986; Kiyotaki *et al.*, 1986; Szomolanyi *et al.*, 1987; Chou *et al.*, 1995). The nucleotide sequence of strain A11 was entirely determined (Albrecht *et al.*, 1992). This viral genome consists of 112 930 bp of L-DNA with 75 tightly packed major open-reading frames. The L-DNA is flanked by some 35 non-coding H-DNA repeats of 1444 bp in tandem orientation. The genome organization is colinear to the human γ_1-herpesvirus EBV. However, the conserved gene blocks are rearranged and the transformation and persistence associated genes of EBV (EBNA, LMP) are lacking (Albrecht *et al.*, 1992). Several open-reading frames of herpesvirus saimiri display strong sequence homologies to known cellular genes, among them are thymidylate synthase, dihydrofolate reductase, complement control proteins, the surface protein CD59, cyclins, and G-protein coupled receptors (reviewed in Albrecht *et al.*, 1992; Albrecht and Fleckenstein, 1994; Fickenscher *et al.*, 1996b). Recently, the viral homologues to IL-17 and to superantigens have been shown to be functional molecules (Rouvier *et al.*, 1993; Yao *et al.*, 1995a,b, 1996; Fossiez *et al.*, 1996).

Moreover, the viral genome codes for two functional inhibitors of apoptosis: open-reading frame 16 belongs to the Bcl-2 family (Nava *et al.*, 1997), and open-reading frame 71 encodes an inhibitor of caspase-8/FLICE recruitment and activation (Thome *et al.*, 1997). The mentioned cell-homologous genes are expressed during lytical virus growth and may play a role in virus replication; expression of these viral genes could not be demonstrated in transformed human T cells (Fickenscher *et al.*, 1996b). Their functional role in T lymphocyte transformation and during the lytic cycle remains to be determined.

The genomic region which is essential for transformation of monkey T lymphocytes and for oncogenicity was mapped to a terminus of the coding L-DNA close to the L-DNA/H-DNA transition (Desrosiers *et al.*, 1984, 1985a, 1986; Koomey *et al.*, 1984; Murthy *et al.*, 1989). Herpesvirus saimiri strains of different subgroups (A, B, C) vary in oncogenicity and terminal genomic sequence (Desrosiers and Falk, 1982; Medveczky *et al.*, 1984, 1989; Biesinger *et al.*, 1990). This genomic region of virus strains from the distinct subgroups encodes different herpesvirus saimiri transformation associated proteins (Stp). The respective proteins of subgroup A and C (StpA and StpC) are able to transform rodent fibroblasts. *StpC488* transformed fibroblasts caused invasive tumours in nude mice (Jung *et al.*, 1991). Moreover, transgenic mice expressing StpC developed epithelial tumours of the salivary gland, bile ducts and thymus (Murphy *et al.*, 1994). *StpA* transgenic animals, in contrast, suffered from peripheral T-cell lymphoma (Kretschmer *et al.*, 1996). All Stp proteins have a hydrophobic carboxy terminus in common. StpC488 contains a stretch of 18 collagen triplet repeat motifs (GPX) and a specific amino terminal sequence of 17 amino acids (Biesinger *et al.*, 1990). StpC was shown to reside in the perinuclear compartment of transformed rodent fibroblasts (Jung and Desrosiers, 1991) and to be phosphorylated in the transformed fibroblasts on a serine residue close to the amino terminus (Jung and Desrosiers, 1992). The amino terminal part of StpC is relevant for its transforming function (Jung and Desrosiers, 1994). The association of StpC with cellular Ras is suggestive for its mechanism of transformation (Jung and Desrosiers, 1995). The neighbouring open-reading frame in subgroup C viruses codes for a protein that specifically interacts with the T-cell-specific tyrosine kinase p56lck. Tyrosine kinase interacting protein (Tip) interferes with Lck-dependent signal transduction and, depending on the assay system, blocks or stimulates its enzymatic activity (Biesinger *et al.*, 1995; Jung *et al.*, 1995a,b; Lund *et al.*, 1995, 1996, 1997; Wiese *et al.*, 1996; Fickenscher *et al.*, 1997). Viral genomes lacking the oncogene region were used as gene expression vectors (Desrosiers *et al.*, 1984, 1985b; Grassmann and Fleckenstein, 1989; Alt *et al.*, 1991; Alt and Grassmann, 1993; Duboise *et al.*, 1996). This approach made it possible to assign the T cell transforming function of the HTLV-1 X region to the Tax protein. Phenotypically, the cultures transformed by *tax* recombinants resembled the known HTLV-1 immortalized cell lines, but did not shed infectious virus (Grassmann *et al.*, 1989, 1992, 1994; Biesinger and Fleckenstein, 1992, Fickenscher *et al.*, 1993).

Primary human T lymphocytes are transformed by wild-type subgroup C strains of herpesvirus saimiri to continuously proliferating T-cell

lines with the phenotype of mature $CD4^+$ or $CD8^+$ cells. Virologists use 'cell line' for cultures growing for long periods with stable properties. In contrast, immunologists describe the clonality as 'cell line' and 'cell clone'. 'Transformation' depicts changes in cell morphology and growth behaviour at the moment, whereas 'immortalization' refers to unlimited proliferation. The growth-transformed human T lymphocytes often remain IL-2 dependent, but do not need a periodic restimulation with antigen or mitogen. They carry multiple non-integrated viral episomes and do not produce virion particles (Biesinger *et al.*, 1992). In transformed human T cells, viral gene expression could not be found from most genomic regions. However, the viral oncogene *stpC/tip* is strongly and inducibly expressed at the transcript and protein level (Fickenscher *et al.*, 1996b). When antigen-specific T cells are transformed by herpesvirus saimiri, the HLA-restricted antigen-specific reaction is retained (Behrend *et al.*, 1993; Bröker *et al.*, 1993; De Carli *et al.*, 1993; Mittrücker *et al.*, 1993; Weber *et al.*, 1993). The transformed T lymphocytes are hyperreactive to CD2 ligation (Mittrücker *et al.*, 1992), maintain early signal transduction patterns of primary cells (Bröker *et al.*, 1993; Mittrücker *et al.*, 1993), express activation surface markers, show inducible cytotoxicity (Biesinger *et al.*, 1992; Klein *et al.*, 1996), and secrete T-helper 1 (Th1) type cytokines (De Carli *et al.*, 1993; Weber *et al.*, 1993). Thus, herpesvirus saimiri provides a novel tool for studying T cell biology (reviewed in Meinl *et al.*, 1995a).

◆◆◆◆◆ PROCEDURES FOR VIRAL LYMPHOCYTE TRANSFORMATION

Propagation and Manipulation of Herpesvirus Saimiri

Owl monkey kidney cells

Epithelial owl monkey kidney (OMK) cells from healthy *Aotus trivirgatus* (owl monkeys) can be kept in primary culture over long periods of time (Daniel *et al.*, 1976a), if they are not contaminated with lytically growing viruses, such as herpesvirus aotus type 1 or 3. The American Type Culture Collection (ATCC) (Rockville, MD) offers strain OMK (637-69) in the 16th passage under catalogue number ATCC CRL-1556. This strain was isolated in November 1970, and later submitted to ATCC by Dr M. D. Daniel, New England Regional Primate Research Center, Harvard Medical School, Southborough, MA, USA. OMK-637 has been used in several laboratories intensively over decades without any hint of viral contamination or latent agents. The monolayer cells are maintained in Earle's minimal essential medium (MEM) or Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), glutamine (350 $\mu g\ ml^{-1}$) and, if desired, antibiotics (100 $\mu g\ ml^{-1}$ gentamycin or penicillin/streptomycin at 120 $\mu g\ ml^{-1}$ each). OMK cell growth does not require exogenous CO_2 supplementation to the atmosphere. The cells are trypsinized and split only once a week onto a doubled area of tissue culture plastic ware. This low ratio maintains the original status of the

primary OMK cells and does not enforce selection for fast-growing subtypes, which are no longer fully permissive. The medium is changed on the fourth day after splitting. The cells should not be used for more than 50 passages. OMK cells are the typical propagation system for herpesviruses of non-human New World primates, such as herpesvirus saimiri, herpesvirus aotus, and herpesvirus ateles (for a review see Fleckenstein and Desrosiers, 1982). It should be kept in mind, that *Aotus trivirgatus* monkeys are an endangered species. If OMK cells have to pass through customs channels, appropriate official documentation according to the CITES convention is needed.

Virus cultures

Traditionally, the tissue culture medium of freshly confluent OMK monolayer cultures is removed on day 2–4 after splitting, and infectious virion suspension is added in a minimal volume (e.g. in 2 ml for a 25-cm^2 flask, in 5 ml for a 80-cm^2 flask). Adsorption is allowed to take place at 37°C for 1–2 h. The monolayer should be covered by the inoculum fluid and should not dry out during this time. Afterwards, medium is added and the incubation is continued. However, we did not observe relevant differences in the time course of infection if the virus suspension was simply added to the culture medium without the adsorption step at low volumes. After 1–14 days, initial cytopathic changes are detectable. Typically, focal rounding of cells is observed. Later, plaques appear, bordered by rounded cells. Several days later, the whole cell layer will be lysed by the virus. When high-titre supernatants are to be prepared, it is essential to inoculate at low multiplicity of infection (0.1–0.5). Ideally, it should last 1–2 weeks, until a complete cytopathic effect (CPE) has developed. A fast CPE at high multiplicity often results in low titres of infectious particles. In such cases, a high proportion of viruses carry repetitive H-DNA only, without coding L-DNA. After completion of the CPE, infected OMK cultures including the OMK cell debris are used as herpesvirus saimiri stocks, without any further treatment. The virus stocks are stable and can be stored at +4°C for several months without loss of infectivity. Small volumes of supernatant should be frozen in liquid nitrogen or at –80°C; the titre may decrease by about one order of magnitude upon freeze–thawing.

The lytic system of OMK cells is used to estimate concentration of infectious virion particles from the supernatant of lytic cultures (see page 580) and also to verify the (non-)producer status of herpesvirus samiri transformed T lymphocytes. Typically, about 10^6 transformed lymphocytes are added to a 25-cm^2 flask (5 ml MEM or DMEM with 10% FCS, but without IL-2) of fresh confluent OMK cells. Co-cultivation allows the infection of OMK cells with small amounts of cell-associated virions from the lymphocytes, as close cell contact is achieved. In many cases, however, the transformed human T lymphocytes are activated by the OMK cell contact, presumably by the CD2/CD58 interaction (Mittrücker *et al.*, 1992). As a side-effect of the activated status, the $CD8^+$ transformed lymphocytes in particular may exhibit a strong non-specific cytotoxic effect on OMK cells.

Thus, the OMK cells are sometimes killed by non-specific cytotoxicity. The cytotoxic activity will decrease during further passage of co-cultivation on fresh OMK cells. If cytotoxicity problems occur, culture supernatants can also be checked for virus particles. For this purpose, large volumes (about 50 ml) of supernatant are collected. Contaminating T cells are removed by low-speed centrifugation (1000 rpm, 10 min). Potentially present virus particles are sedimented from the supernatant by high-speed centrifugation in a Sorvall centrifuge at 20 000 rpm and 4°C for at least 4 h in a SS34 rotor using screw-capped tubes (35 ml; Nalgene). The sediment is resuspended in medium and transferred onto fresh OMK cultures. Traditional co-cultivations included three passages, which takes about 6 weeks. We now have good experience with virus isolation attempts, if the culture medium was simply exchanged every fourth day for 6 weeks with no further passaging. Under these conditions, low amounts of infectious particles are easily detected. Questionable or weak CPEs can be confirmed by transferring an aliquot of sterile filtered supernatant to a fresh OMK culture, where a typical CPE should be visible after few days. If there was virus growth only after several passages, very low titres of infectivity have been amplified. Several weeks after infection, virion production from human T lymphocyte cultures can no longer be demonstrated (Biesinger *et al.*, 1992; Fickenscher *et al.*, 1996b). The sensitivity of the co-cultivation method was estimated to be one virus-producing cell per 10^6 cells (Wright *et al.*, 1976).

Virus strain C488 (Desrosiers and Falk, 1982; Biesinger *et al.*, 1990) was submitted to ATCC (Rockville, MD) after only a few passages on OMK cells. The virus is available under order numbers VR-1396 and VR-1414. Moreover, ATCC offers virus strain S295C, which does not belong to subgroup C. This subgroup B virus is unable to transform human T cells, but immortalizes marmoset monkey cells with low efficiency (Meléndez *et al.*, 1968; Fleckenstein *et al.*, 1977).

Virion purification

The supernatant and the cell remnants from lytic virus cultures in OMK both contain high amounts of virion particles (about 10^9 virion particles per ml in the supernatant). Cultures with a complete CPE (e.g. 1 or 2 l) are bottled into Sorvall-GSA centrifuge flasks (with canted necks, to avoid any spilling) at 200 ml each. A first centrifugation is carried out for 15 min at 4°C and 3000 rpm to collect the cell-associated fraction of the virus. The cell sediment is resuspended in 3 ml hypotonic 'virus standard buffer' (VSB: pH 7.8, 50 mM Tris, 12 mM KCl, 5 mM EDTA). The supernatant is transferred to clean GSA bottles and spun for a second time at high speed (12 000 rpm) and 4°C for 4 h. Usually, clearly visible sediments are found. Virions are resuspended in 3 ml VSB. Sucrose gradients are prepared in ultra-clear Beckman ultracentrifuge tubes (SW27 or SW28) using a gradient mixer with 15 ml of 30% (w/w) sucrose in VSB solution (stirring) close to the outlet and 16 ml of 15% (w/w) solution in the distal container. The rotor and the buckets are cooled to 4°C prior to centrifugation. Both virus and cell-debris suspensions are dounced in a glass homogenizer by

20 strokes. About 3 ml of the homogenized material can be loaded on one gradient. The buckets are weight adjusted with VSB and centrifuged in a SW27 or SW28 rotor for 30 min at 4°C and 20 000 rpm. In case the virus band is not clearly visible, a strong focused apical light source should be used. The band material is collected into a Beckman SW27 or SW28 polyallomer tube, diluted with VSB, and spun for 90 min at 4°C and 17 000 rpm. Alternatively, 35-ml Nalgene tubes with a screw cap can be used in a Sorvall SS34 rotor at 20 000 rpm and 4°C for 4 h. The virions are then resuspended in a small volume (e.g. 100–500 µl) of VSB. This method is based on the procedures described by Fleckenstein and Wolf (1974) and Fleckenstein *et al.* (1975). The small final volume should contain nearly all infectious particles from the starting material and is concentrated by several orders of magnitude. Therefore, special care should be taken for the biosafety of the laboratory staff. Large-scale virion purifications of oncogenic herpesvirus saimiri strains should be performed in a P3 facility.

Purification of virion DNA

Purified virion DNA of oncogenic herpesvirus saimiri strains is infectious. When virion DNA was injected intramuscularly into marmoset monkeys, animals died from lymphoma after a short time (Fleckenstein *et al.*, 1978). Large-scale purification of virion DNA starts with concentrated virion suspensions as described above. Sodium lauryl sarcosinate (200 µl, 20%) is added to 1.8 ml purified virions in VSB. The lysis of virions is performed for 1 h at 60°C. The lysate (2 ml) is diluted with 6 ml VSB, and 10 g CsCl is added. The solution should have a refraction index of 1.412 at 25°C. The DNA/CsCl solution is loaded without ethidium bromide in 50 TI Beckman quickseal tubes (polyallomer), which are then filled to completion with paraffin. The gradients are spun at 35 000 rpm and 20°C for 60 h. Two syringe needles are placed into the gradient tube: the first one on top into the paraffin to allow pressure compensation, and the second one at the bottom. The flow rate can be reduced, when a syringe filled with paraffin is plugged into the upper needle. About 20 fractions of 10 drops each are collected in Eppendorf caps. A portion (1 µl) of each fraction is diluted to 10 or 15 µl with water and coloured loading buffer, and run slowly on a conventional agarose test gel. One single clean high molecular weight band at limiting mobility should be visible. The pooled DNA-containing fractions are dialysed against 20 mM Tris pH 8.5 and extracted with phenol/chloroform/isoamylalcohol and chloroform/isoamylalcohol, ethanol precipitated, and resuspended in water. Concentration is determined by spectrophotometry. The yields are about 40 µg from 200 ml supernatant from infected cultures. Virion DNA isolated from the cleared supernatant will be rather pure (less than 5% contamination by cellular DNA). The cell-bound virus may lead to varying quality and yields of DNA. However, large amounts of pure virion DNA are often obtained from the cell fraction. These procedures are modified from Fleckenstein and Wolf (1974), Fleckenstein *et al.* (1975), and Bornkamm *et al.* (1976).

A short protocol is useful for the preparation of virus DNA on a small scale. When only small amounts of virion DNA are needed, 1.0 ml cell free

supernatant is centrifuged at 13000 rpm in a cooled benchtop microcentrifuge for several hours. It is recommended that screw-cap Eppendorf tubes and a rotor with an appropriate lid are used in order to exclude aerosol formation. Sodium dodecyl sulfate (SDS) buffer (100 µl) is added (1% SDS, 50 mM Tris-HCl pH 8.0, 100 mM EDTA, 100 mM NaCl, 1 mg ml^{-1} proteinase K) to the virus sediment. The lysates are incubated at 56°C for 2 h, diluted with water to 300 µl, phenol extracted, chloroform extracted, and ethanol precipitated. The yield from 1.0 ml is about 50 ng and sufficient for several Southern analyses (modified after Grassmann *et al.*, 1989).

Virus titration

The limiting dilution method is the simplest way to estimate virus titres. OMK cells are trypsinized and split by 1:5 per area (e.g. into 24-well dishes) and incubated under CO_2. The cell-free virus suspension is diluted in DMEM at 10^{-4} to 10^{-9}. To each 24-well plate 1 ml of virus dilutions is added 1 day after splitting. It is important to perform the assay at least in triplicate and to run control cultures. The plates are observed for at least 14 days. The progress of the CPE should be monitored daily.

The more laborious plaque assay is also based on serial dilution. Methylcellulose (4.4 g; e.g. Fluka 64630 Methocel MC 4000) is suspended in 100 ml H_2O in a 500-ml bottle and autoclaved (45 min, 121°C). One-hundred millilitres of 2 × DMEM, 20 ml FCS (heat inactivated), antibiotics and glutamine (350 µg ml^{-1}) is added. The mixture is stirred at 4°C overnight. Subconfluent OMK cultures in 6-cm dishes are each infected with 1 ml of cell-free virus dilutions (10^{-3} to 10^{-8} in DMEM) to three dishes each, 1 day after splitting the cells. After 1 h at 37°C, the supernatant medium is aspirated, and 5 ml of 2% methylcellulose in DMEM is added. The cultures are fed with 3 ml of 2% methylcellulose in DMEM after 1 week, if needed. If this approach is used for virus plaque purifications, single plaques are picked in about 10 µl with a pipette tip and transferred to a fresh OMK culture. To exclude clumping of virus particles, the supernatant should be sterile filtered before starting the plaque cloning cultures. This will allow virus cloning at a low rate of contamination by other virus clones. If the recombinants carry a resistance gene, G418 or hygromycin should be added into the plaque culture medium. For titre estimations, the medium is poured off carefully after plaque formation has been observed. The cells are washed mildly with PBS and stained with 1% crystal violet in 20% ethanol/80% water (v/v). Subsequently, the surplus dye is washed out carefully with water without destroying the cell layer. The cells are air dried, and the plaques are counted. Routine OMK cell cultures should reach titres between 10^5 to 10^7 plaque forming units (pfu) per ml (Daniel *et al.*, 1972).

Demonstration of episomes and virion DNA

In order to demonstrate and distinguish persistent non-integrated episomal (slowly migrating) or lytical linear (higher mobility) viral

DNA, *in situ* lysis gel electrophoresis can be applied. The method was first described by Gardella *et al.* (1984). A simplified version of that protocol is presented here (modified after Ablashi *et al.*, 1985). Typical examples of 'Gardella gels' are given in various reports (Gardella *et al.*, 1984; Ablashi *et al.*, 1985; Biesinger *et al.*, 1992; Bröker *et al.*, 1994; Meinl *et al.*, 1997). The gel system is a vertical 1% agarose gel in 1× Tris Borat EDTA (TBE) (gel size 20×20 cm, 0.5 cm thick) at 4°C. The wells have 0.5×0.5 cm area and are 1 cm deep; they are separated by 0.3–0.5 cm agarose teeth. The thick gel tends to slip out from the gel plates during assembly of the gel apparatus. A large 3% agarose block is put into the lower buffer chamber, so that the gel and the gel plates can rest on it. The gel system is placed into a refrigerator or cold room; the upper chamber is left without buffer. TBE (1×) for the chambers is precooled. Frozen aliquots of buffers A and B (composition listed below) without enzymes are prepared in advance, as Ficoll tends to be very sticky. Enzymes (RNase, protease K) are added just before use. About 2×10^6 lymphocytes (e.g. transformed with herpesvirus saimiri C488) are washed in PBS, resuspended in 50 µl blue sample buffer A (200 mg ml^{-1} Ficoll 400, 1× TBE, 0.25 mg ml^{-1} bromphenol blue, 50 µg ml^{-1} RNase A) and loaded into the very bottom of the slots. As controls, conventional λ-marker (*Hind*III) and a small amount of virions are used, the latter being obtained by centrifuging 1.0 ml virus suspension at 13 000 rpm in a cooled benchtop centrifuge, as described for the miniprep protocol for virion DNA. Such a small virion preparation should be sufficient for several gels. The controls are also loaded in buffer A. Green buffer B (120 µl per sample; 50 mg ml^{-1} Ficoll 400, 1× TBE, 1% SDS, 0.25 mg ml^{-1} xylene cyanole, 1 mg ml^{-1} protease K) is laid over the sample, nearly filling the gel slots. Some 1× TBE buffer is added carefully to fill the slots completely, and more buffer is added to fill the upper chamber. Electrophoresis is performed slowly at 4°C. Proteins and RNA are allowed to degrade enzymatically during a first period of at least 3 h at 10 V. Subsequently, electrophoresis is continued for at least 12 h at 100 V, until xylene cyanole (green) reaches the bottom of the gel. The agarose gel is stained in TBE containing ethidium bromide, and photographed. However, ethidium bromide staining is usually not sufficient to demonstrate episomal bands. The DNA is transferred onto a nylon membrane (e.g. Hybond, Amersham) using the alkali transfer protocol. Viral DNA bands will be visualized by stringent hybridization and subsequent autoradiography. There are several alternative choices of hybridization probe. If maximal sensitivity is required, a cloned H-DNA fragment should be used (Bankier *et al.*, 1985). H-DNA is highly repetitive in the non-integrated episomes and amplifies the viral signals. If higher specificity is needed (e.g. to prove that the correct virus strain has been used), a fragment from the variable genomic end should be applied (Biesinger *et al.*, 1990). If band intensities from cultures infected with different virus strains are to be compared, it might be advantageous to take a probe from a less variable genomic region. Insert DNA as well as flanking sequences should be used to probe for recombinant viral vectors.

Foreign genes can be inserted efficiently behind open-reading frame 75 into the terminal genomic junction region of herpesvirus saimiri L-DNA and H-DNA, where no hint of viral transcription has been found (Bankier *et al.*, 1985; Stamminger *et al.*, 1987). The neomycin resistance marker gene was inserted into that position by Grassmann and Fleckenstein (1989). Cloning into herpesvirus saimiri is achieved via homologous recombination. As herpesviruses are assumed to replicate according to the rolling circle model, a single cross-over suffices for insertion of the foreign gene (Grassmann *et al.*, 1989). Therefore, the respective recombination plasmids have to provide in series the terminal genomic sequences, the gene of interest to be inserted, and a selection marker. Two kb of homologous terminal sequences were sufficient for homologous recombination. Alt *et al.* (1991) constructed the plasmid pRUPHy containing 2 kb of homologous sequence, a multilinker stretch for the insertion of the gene of interest, and the hygromycin-B resistance gene which was under the transcription control of the human cytomegalovirus major immediate early enhancer–promoter and had the SV40 termination signals. The targeting plasmid (4 μg) is linearized upstream of the viral segment (*Sma*I and *Nsi*I sites of plasmid pRUPHy), mixed at about 200-fold molar excess with purified infectious virion DNA (200 ng), and co-transfected into permissive OMK cells by the calcium phosphate method. Virion DNA is prepared on a small scale from the supernatant at complete CPE (after 2–3 weeks), and tested by Southern hybridization. Clean recombined virus stocks are obtained using selection media (200 μg ml^{-1} hygromycin-B) and by plaque purification.

In an alternative protocol, the linearized recombination plasmid is transfected into OMK cells using lipofectamine (Gibco BRL). One day after transfection, the cells are infected with wild-type virus. After completion of the CPE in such cultures, recombinant viruses are enriched in serial passage under increasing concentrations of the selection drug, such as G418 at 100, 200, and finally 300 μg ml^{-1}. It is important to use a low multiplicity of infection (e.g. 0.1–0.5) in order to avoid wild-type virus overgrowing the culture before selection can take place. The CPE should take at least 10 days to reach completion, in order to achieve efficient selection. When the recombination has been proven by Southern hybridization from such supernatants, plaque purification of sterile-filtered material is performed in the presence of 200 μg ml^{-1} G418. Sterile-filtration removes virus clumps from the suspension and enhances cloning efficiency. The plaque purification procedure is described on page 580. Under selection pressure, herpesvirus saimiri recombinants with G418 resistance were shown to infect a broad spectrum of cell types (Simmer *et al.*, 1991). Cloned virus stocks may be used to infect T cells to introduce foreign genes, such as the transforming genes of HTLV-1 (Grassmann *et al.*, 1989, 1992).

The transformation-associated region of herpesvirus saimiri at the other genomic end has also been used for vector purposes. As the transformation-associated genes are not essential for virus replication, they can be replaced

by foreign genes. Such an approach was first used for the expression of bovine growth hormone (Desrosiers *et al.*, 1985b). Recently, virus mutants have been constructed that carry the genes for secreted alkaline phosphatase or green fluorescent protein instead of the transforming region. This vector system uses unique *Asc*I and *Not*I restriction enzyme sites introduced into the viral genome. A major advantage of this system is the easy manipulation by restriction enzyme digestion and conventional ligation into purified virion DNA (Duboise *et al.*, 1996). The use of green fluorescent protein and secreted alkaline phosphatase provides two alternative methods for screening for recombinants. In the first case, the enzyme is detected from the supernatant by a simple enzyme-linked immunosorbent assay (ELISA). In the second case, fluorescent plaques are detected by conventional fluorescence microscopy (Duboise *et al.*, 1996).

A third method has been developed using overlapping cosmids of C488 virus DNA (Ensser *et al.*, 1998). Mutations or insertions can be performed easily by conventional cloning procedures in *Escherichia coli*. Appropriate sets of cosmids are co-transfected into OMK cells using lipofectamine. By homologous recombination, overlapping cosmids reconstitute replication-competent virus DNA. The resulting virus populations are free of contaminating wild type virus (Ensser *et al.*, 1998).

Growth Transformation of Human T Cells by Herpesvirus Saimiri

Infection of primary human T lymphocytes with herpesvirus saimiri subgroup C strains, but not with strains of subgroups A or B, yields continuously proliferating mature T-cell lines that exhibit the $CD4^+/CD8^-$ or the $CD4^-/CD8^+$ phenotype (Biesinger *et al.*, 1992; Fickenscher and Fleckenstein, 1994; Meinl *et al.*, 1995a). Permanently growing T-cell lines have been obtained from primary cells from various sources. The degree of purity did not influence the outcome. Polyclonal preparations of mononuclear cells from adult peripheral blood or from cord blood, from thymus or bone marrow, as well as characterized T-cell clones, or flow-cytometry sorted T cells are suitable for the procedure. The CD4/CD8 and Th1/Th2 phenotypes do not influence the susceptibility to growth transformation. Transformed cell lines with αβ-TcR represent a broad variety of Vβ chains (Fickenscher *et al.*, 1996a). The transformation of bulk cultures is polyclonal (Behrend *et al.*, 1993; Fickenscher *et al.*, 1996a; Saadawi *et al.*, 1997). Although γδ cell lines were never obtained when large amounts of polyclonal cells were infected, they were generated either after lysis of αβ-T cells, or from small cultures at microwell scale, or even upon differentiation of virus infected $CD34^+$ precursor cells (Yasukawa *et al.*, 1995; Klein *et al.*, 1996; Pacheco-Castro *et al.*, 1996; Fickenscher *et al.*, 1997). Primary or proliferating cells with good viability and with the morphology of activated T lymphocytes are needed. The primary cells may be purified by applying Ficoll density gradients (1.077 g ml^{-1}) (Biochrom, Berlin) or the dextran sedimentation protocol (selective sedimentation of erythrocytes at 37°C and 1.2% dextran 250 000 (w/v)/30 mM NaCl for 45 min). Prestimulation of fresh primary cells for 1 day with PHA (0.5–10 μg ml^{-1}) (Murex/Wellcome) or OKT-3

(10–200 ng ml^{-1}) (Ortho) has no significant advantage over the use of untreated samples. Whether or not mitogenic stimulation should be performed depends mainly on the number of available cells. Antigen-dependent T-cell clones should be restimulated with antigen and feeder cells 3–5 days prior to infection. If the antigen specificity of an established clone is not known, the cells should be stimulated by mitogen 3–5 days before infection. The T-cell cultures are kept at a density of about 1 $(0.5–1.5)\times10^6$ cells ml^{-1}.

Different types of cell culture plastic ware have been tested. The cells retain best viability in 25-cm^2 flasks (3–10 ml with 3×10^6 to 10×10^6 cells), 24-well plates (1–2 ml with 1×10^6 to 2×10^6 cells), or 96 round-bottomed microwell plates (100–200 µl with 10^4 to 10^5 cells). The culture flasks should be incubated with a slightly elevated top (at an angle of about 15°) to allow close cell contact in the lower edge of the culture flask. Various media formulations have been used. Lines CB-15, PB-W, Lucas (Biesinger *et al.*, 1992) and CB-23 (Nick *et al.*, 1993) have been isolated without prestimulation, without IL-2 supplementation, and with a standard medium formulation (RPMI 1640 80%, FCS 20%, 50 µM 2-ME, glutamine, gentamycine). During further experiments, the addition of supplements enhanced the transformation efficiency considerably. The quality of FCS batches, even from the same supplier, varies remarkably. It seems to be very important to compare different serum batches and to take a batch with low endotoxin level. FCS is inactivated at 56°C for 30 min in order to avoid mainly complement-dependent problems with both virus and lymphocytes. The addition of IL-2 (20–50 U ml^{-1} in the medium stock) activates cell proliferation and enhances the transformation frequency. As usually about one-third of the culture medium is added or replaced twice a week, a final concentration in the culture of less than 20 U ml^{-1} IL-2 is presumed. Comparison of many different IL-2 batches from different suppliers revealed consistently in our experiments that pretested batches with low endotoxin levels of the human recombinant IL-2, isolated from *E. coli* and offered by Boehringer Mannheim (catalogue Nos 1011456 and 1147528), are very active and provide optimal conditions for a high transformation efficiency. For financial reasons this could be applied mainly in the initial transformation procedure itself and for maintaining small culture volumes. When stable growth has been established and when large cell culture volumes of transformed lines are required, use of the less expensive and less active Proleukin at 50–100 U ml^{-1} in the medium stock is recommended. It is a human recombinant IL-2 for clinical use, which carries 1-des-Ala and 125-Ser amino acid substitutions and is available from Chiron (Ratingen, Germany). Efforts to enhance transformation efficiencies led to the observation that adding 45% of 'CG medium' was beneficial. CG (cell growth) medium is a defined synthetic medium, which was originally designed for the serum-free culture of B-cell hybridomas (Lang *et al.*, 1992) (available exclusively from Vitromex, Selters, Germany; composition listed in Fickenscher and Fleckenstein, 1994). Several types of synthetic media were tested as supplement, but only CG medium (Vitromex) and AIMV (Gibco/BRL, Eggenstein, Germany) improved transformation efficiency significantly in our experiments. CG medium allows the metabolic activity to be judged

by colour, whereas there is little colour difference using AIMV medium. Unfortunately, Gibco/BRL does not provide a list of the ingredients to customers. Using pretested IL-2 (Boehringer Mannheim) and CG medium, about 90% of primary T-cell cultures were transformed to antigen- and mitogen-independent growth. The failure rate of about 10% is mostly explained by trivial factors, such as low viability of the cells prior to infection. In our experience, the optimal medium formulation is: RPMI 1640 45%, CG 45%, FCS 10%, glutamine, gentamycine (if desired) and 20–40 U ml^{-1} IL-2 (Boehringer Mannheim). In order to block autoxidation of thiols and the resulting toxicity, the addition of 1 mM sodium pyruvate (Gibco-BRL 043-01360, 100× stock solution at 100 mM), 50 μM α-thioglycerol (Sigma M-6145, 1000× stock solution at 50 mM), and 20 nM bathocuproine disulfonic acid (Sigma B-1125; 1000× stock solution at 20 μM) is advantageous, but not essential (M. Falk and G. Bornkamm, personal communication). α-Thioglycerol is a more stable alternative to β-mercaptoethanol. Copper ions, which are relevant for autoxidation, are chelated by bathocuproine disulfonic acid. Addition of sodium pyruvate reduces H_2O_2 concentrations (O'Donnell-Tormey *et al.*, 1987). Most experience in T cell transformation is based on using the herpesvirus saimiri strain C488 (Biesinger *et al.*, 1992). However, different strains, called C484 (Desrosiers and Falk, 1982; Medveczky *et al.*, 1984, 1989, 1993a; Biesinger *et al.*, 1992; Fickenscher *et al.*, 1997) and virus strain C139, are also able to transform human T cells, at least to a certain degree and for certain phenotypes. Strain C-139 is an isolate from our laboratory (Klein *et al.*, 1996; Fickenscher *et al.*, 1997). C488 seems to be the most reliable herpesvirus saimiri strain for transforming human T cells (Fickenscher *et al.*, 1997). Typically, 10% (v/v) of infectious OMK supernatant is added to a vital lymphocyte culture, for example 500 μl of 10^6 pfu ml^{-1} (5×10^5 pfu) is added to 5 ml culture with 5×10^6 cells ml^{-1}. During the following weeks, and sometimes months, the cells need patient care; medium should be partially exchanged at regular intervals (about twice a week). Good negative controls are uninfected cells and cultures infected with a virus strain of the same titre, such as strain 11 (subgroup A) or SMHI (subgroup B), which are not able to transform human T cells. During the first weeks of culture, normal growth of T lymphocytes is observed. This normal mitotic phase should not be misinterpreted as transformation events and it may last for up to several months, before the viral infected T cells start to proliferate rapidly. About 10^5 viable cells at least are necessary to obtain a transformed culture (Fickenscher *et al.*, 1997). The efficiency seems to vary considerably between different donors. When using a few million vital cells for infection, the efficiency rate reaches 90%. Several criteria have been found to indicate a transformed phenotype: (1) a doubling of the cell number constantly 1–4 times a week over several months, independent of antigen and mitogen; (2) the morphology of T lymphoblasts, enlargement of cells, good contrast upon microscopy and irregular shape; (3) death of control cultures that have been treated identically to the transformed ones; (4) persistence of viral non-integrated episomes without virion production (PCR, Gardella gel, and co-cultivation test on OMK); and (5) CD2 hyperreactivity against membrane-bound CD58, or cross-linked CD2 antibodies, or

foreign cells (Mittrücker *et al.*, 1992), and non-specific cytotoxicity. Cultures which seem to be growth transformed can often be subjected to a gradual withdrawal of the CG medium component. Addition of 10 mM HEPES pH 7.4 (Gibco/BRL) helps to avoid cell degeneration caused by low pH. In $CD4^+$ cells, exogenous IL-2 supplementation could also be reduced gradually, and can even be terminated in several cases. However, withdrawing IL-2 from $CD8^+$ transformed cell lines for longer periods was not successful in our experiments.

Transformation by Human T Cell Leukaemia Virus Type I

Human T cell leukaemia virus type 1 (HTLV-1) has been used extensively for immortalizing human T cells. Here, the method described is based on an excellent report by Nutman (1997). HTLV transformed T cells do release virus particles. Moreover, HTLV is a human tumour virus, and thus must be handled under containment level P3 conditions. Mononuclear cells from cord blood or adult peripheral blood or T-cell clones are applied at 10^6 cells ml^{-1} in complete RPMI-1640 medium with 10–100 U ml^{-1} recombinant IL-2. The cells are activated with mitogen (PHA, Murex/Wellcome) or with the specific antigen, and incubated in 24-well plates for 2–3 days. Activated cells are seeded at 10^6 cells ml^{-1} in 24-well plates. The HTLV-1 producer cells (10^6 cells ml^{-1}; e.g. MT-2 or HUT-102, available from ATCC as TIB-162) are irradiated at 6 krad in a γ-irradiator (^{137}Cs source, e.g. Gammacell 1000). Alternatively, the cells are inactivated with 50 $\mu g\ ml^{-1}$ mitomycin C for 1 h, followed by extensive washing with buffered saline. One million inactivated HTLV producer cells are added to 10^6 stimulated untransformed cells. The co-cultures are incubated for 2–6 weeks with feeding IL-2 containing medium twice weekly. After 6 weeks, rapidly growing cells should be observed. In many cases, such cells are already IL-2 independent. The co-cultivation is more efficient and reliable in comparison to infection with cell-free supernatants. HTLV-transformed T cells express CD25/IL2Rα chain and HLA-DR molecules abundantly on their surface. After few months of culture, HTLV-transformed human T cells tend to lose IL-2 dependance, cytotoxicity and TcR expression (Inatsuki *et al.*, 1989; Yssel *et al.*, 1989).

Immortalization of Human B Lymphocytes

In contrast to viral transformation of human T lymphocytes, the B-cell transformation by EBV is a widely applied, reliable and easy procedure, which does not require much care with regard to the cultures. The protocol described here is based on own experience and on the recommendations of Tosato (1997). Like the viruses used in the other protocols, EBV is considered a human pathogen (containment level P2). EBV-negative persons should not have access to EBV-containing cultures. One should bear in mind that EBV is easily reactivated from most EBV-transformed lymphoblastoid cell lines.

In order to generate EBV stocks, exponentially growing cells of the EBV-transformed marmoset producer cell line B95-8 (ATCC CRL-1612) are

seeded at 1×10^6 cells ml^{-1} in RPMI 1640 with 10% FCS, glutamine and antibiotics. After 3 days of culture, the cells are sedimented at low speed (1000 rpm, 10 min). The supernatant is sterile filtered and stored in portions at –80°C. Such supernatants contain up to 1000 transforming units per ml.

Fresh gradient-purified peripheral blood cells (10^7) in 2.5 ml complete RPMI medium are infected by addition of 2.5 ml thawed supernatant from B95-8 cells (as described above). After 2 h at 37°C, 5 ml complete medium is added. EBV-specific cytotoxic T lymphocytes may overgrow such cultures. For this reason, cyclosporin A can be added to such cultures at a final concentration of 0.5 μg ml^{-1}. During the following 3 weeks, the cultures do not require much care. Once weekly, some medium may be exchanged (1/5 volume) without removing cells. After 3 weeks, and depending on the metabolic activity, the cells can be expanded in complete medium. In most cases, rapid proliferation is established in a short time, which allows splitting of the cells twice weekly at ratio of 1 : 2 per volume. The resulting lymphoblastoid B-cell lines (Nilsson and Klein, 1982) are latently infected by EBV, and in many cases can be induced to production of virus antigens and particles.

◆◆◆◆◆◆ PHENOTYPE OF TRANSFORMED T CELLS

Persistence of Herpesvirus Saimiri

Herpesvirus saimiri, a monkey virus, can infect human cells. An early analysis of herpesvirus saimiri strain SMHI revealed weak productive growth on primary human fetal cells (Daniel *et al.*, 1976b). Selectable herpesvirus saimiri recombinants, derived from strain A11, were later used to study the spectrum of cells that can be infected by the virus (Simmer *et al.*, 1991). A broad range of epithelial, mesenchymal and haematopoetic cells became infected and carried non-integrated episomal DNA of the recombinant viruses. The pancreatic carcinoma line PANC-1 and human foreskin fibroblasts even produced infectious virus under selection conditions. These findings suggest that the receptor used by herpesvirus saimiri is widely distributed among various tissues. The receptor seems to be well conserved, as rabbit T cells can also be infected and transformed by herpesvirus saimiri strains (Ablashi *et al.*, 1985; Medveczky *et al.*, 1989). Cell lines that had been infected with the recombinants under selection pressure retained the viral non-integrated episomes after withdrawal of the selecting drug for long time periods. The lack of counterselection against cells with persisting viral episomes may suggest that the virus persists with mostly suppressed viral gene expression (Simmer *et al.*, 1991). This model is also supported by the observations that the persisting non-integrated viral episomes are heavily methylated (Desrosiers *et al.*, 1979) and may carry extensive genomic deletions (Schirm *et al.*, 1984). These observations were made using mainly herpesvirus saimiri strain A-11. It is likely that they are also valid for subgroup C strains and for transformed human T cells. Although monkey T lymphocytes produce herpesvirus saimiri

particles in many cases, it was not possible to isolate virus from transformed human T-cell cultures that carry non-integrated viral episomes in high copy number (Biesinger *et al.*, 1992). Even after treatment with phorbol esters, nucleoside analogues and other drugs known to cause reactivation of other viruses like EBV, or after specific or non-specific stimulation of the T cells, no virion production could be demonstrated (Fickenscher *et al.*, 1996b). Nevertheless, it will be difficult to provide formal proof that the virus can never be reactivated from transformed human T lymphocytes.

Viral Gene Expression in Human T Cells Transformed by Herpesvirus Saimiri

Numerous genomic regions of the virus have been used to search for viral transcripts in the transformed cells. Viral transcription could not be demonstrated from any tested genomic region, except from the genomic terminus, where the viral oncogene *stpC/tip* is transcribed (Fickenscher *et al.*, 1996b). An exception is formed by the non-coding viral U-RNA genes (herpesvirus saimiri U-RNA (HSUR)). The HSUR RNAs are expressed abundantly in presence of viral DNA, similarly to the EBER RNAs of EBV (Albrecht and Fleckenstein, 1992; Albrecht *et al.*, 1992; Biesinger *et al.*, 1992; Myer *et al.*, 1992). In contrast to transformed human T cells, virion-producing monkey lymphocytes show broad viral transcription. In transformed human T cells, the mRNA of the viral oncogene *stpC/tip* is subjected to cellular regulation. Two proteins are translated from the bicistronic transcript. The cytoplasmic phosphoprotein StpC has a predicted size of about 10 kDa and migrates at 21 kDa on SDS protein gels (Jung and Desrosiers, 1991; Fickenscher *et al.*, 1996b). p40Tip is expressed at extremely low levels and was detectable only after Lck immunoprecipitation with subsequent Lck phosphotransferase assay (Biesinger *et al.*, 1995; Jung *et al.*, 1995a,b; Lund *et al.*, 1995, 1996, 1997; Wiese *et al.*, 1996; Fickenscher *et al.*, 1997). Similarly to the reported observations from virus strain C488, the homologous *stpC484* transcript is expressed in transformed cells (Geck *et al.*, 1990, 1991; Medveczky *et al.*, 1993b). In strain C484, a tricistronic transcript has been described from tumour cells, encoding dihydrofolate reductase and the transformation-associated genes (Whitaker *et al.*, 1995). Most likely this transcript is correlated with lytic conditions (Fickenscher *et al.*, 1996b).

The replication origins of herpesvirus saimiri have not yet been clearly defined. Preliminary work by Schofield (1994) assigned the origin of lytic replication to the region uspstream of the thymidylate synthase gene. Kung and Medveczky (1996) identified a viral DNA fragment that permits stable episomal replication in transformed T cells. This observation, however, is not sufficient to define the plasmid replication origin. There is pronounced sequence variation between virus strains in this region, and viral deletion mutants of this region have been described, which are still capable of episomal persistence. It is noteworthy that strain C139 persists at low copy number in comparison to the high copy number of all other previous studies on herpesvirus saimiri (Fickenscher *et al.*, 1997).

The oncoprotein StpC488 was shown to transform rodent fibroblasts, leading to invasive tumours in nude mice (Jung *et al.*, 1991). *StpC* transgenic mice (Murphy *et al.*, 1994) develop epithelial tumours, namely salivary gland adenomas and adenocarcinomas, and bile duct and thymus epitheliomas. However, when the transformed human T lymphocytes expressing *stpC/tip* were tested for tumorigenesis in nude or SCID mice in a conventional implantation experiment, no tumour formation could be observed (Huppes *et al.*, 1994). In macaque monkeys transfused with autologous transformed T cells, no leukaemias or lymphomas developed, and the animals were protected from disease after intravenous challenge infection with high-titre wild-type virus (Fickenscher *et al.*, unpublished results). In naive macaques, herpesvirus saimiri C488 causes an acute disease that is lethal within 2 weeks. Most probably, it is a peracute form of polyclonal T cell leukaemia, which has not been described in humans (Fickenscher *et al.*, unpublished data; Jung and Desrosiers, personal communication).

Immunological Phenotype of Herpesvirus Transformed Human T Cells

The phenotype of human growth-transformed T lymphocytes has been surveyed by Fickenscher and Fleckenstein (1994) and Meinl *et al.* (1995a). Human T lymphocytes, which are transformed to stable growth by herpesvirus saimiri C strains, are mostly IL-2 dependent and do not need restimulation with antigen or mitogen. Cell number increases by factors of 2–4 per week. The cells show the morphology of T blasts with irregular shape and express surface molecules that are typical for activated mature T lymphocytes. The cell lines exhibit the $CD4^+/CD8^-$ or the $CD4^-/CD8^+$ phenotype. Mixed populations may occur when polyclonal populations are infected. The surface antigens CD2, CD3, CD5, CD7, CD25 (IL-2R α-chain), CD30, MHCII and CD56 (natural killer (NK) marker) are expressed, while the NK markers CD16 and CD57 are absent (Biesinger *et al.*, 1992). The RO and RB isoforms of the membrane-bound phosphatase CD45, which are typically found on mature memory T cells, were both present on the $CD4^+$ transformed T lymphocytes. The $CD8^+$ transformed cell lines, however, also expressed the CD45 isoform RA, which is typical of naive T cells and their precursors (B. Simmer and E. Platzer, unpublished observations).

Cytokines are produced by the transformed human T blasts after activation. IL-2 (Mittrücker *et al.*, 1992) and IL-3 (De Carli *et al.*, 1993) are secreted by the cells in response to mitogenic or antigenic stimuli. Antibodies against the IL-2R α-chain (Mittrücker *et al.*, 1992) and against IL-2 and IL-3 (De Carli *et al.*, 1993) suppressed the growth rate; both cytokines seem to support autocrine growth. IL-4 and IL-5 are secreted only at low rates by transformed Th2 cells (De Carli *et al.*, 1993). Transformed $CD4^+$ T cells secrete γ-interferon (IFN-γ), tumour necrosis factors α and β (TNFα, TNFβ) and granulocyte–macrophage colony stimulating factor (GM-CSF) after specific or unspecific stimulation

(Bröker *et al.*, 1993, De Carli *et al.*, 1993; Weber *et al.*, 1993; Meinl *et al.*, 1995b). Both, Th1 and Th2 clones were transformed by herpesvirus saimiri. The cytokine pattern of the Th1 clones was enhanced, while the profile of Th2 clones switched to a mixed phenotype after transformation (De Carli *et al.*, 1993). The viral transformed human T cells show an inducible non-specific cytotoxic activity. When tested on K562 cells, $CD8^+$ lines and to a lesser extent $CD4^+$ cells, showed NK-like cytolytic activity (Biesinger *et al.*, 1992). The lectin-dependent cytolytic activity of Th1 clones against P815 target cells was enhanced after transformation, while Th2 clones showed this activity only in the transformed state (De Carli *et al.*, 1993). The cytotoxic activity of a transformed γδ-T-cell clone on K562 was inducible by stimulation with IL-12 (Klein *et al.*, 1996). Herpesvirus saimiri transformed T cells are capable to deliver non-specific B cell help via membrane-bound TNFα or via CD40 ligand (Del Prete *et al.*, 1994; Hess *et al.*, 1995; Saha *et al.*, 1996a).

The karyotypes of a series of cell lines have been analysed in detail and found to be normal (Troidl *et al.*, 1994). When early signal transduction properties of the transformed cells were compared with those of the uninfected parental cells, no significant differences were encountered. After stimulation with IL-2, anti-CD3 and/or anti-CD4, similar patterns of tyrosine phosphorylation and calcium mobilization were observed in primary clones or in transformed lines. In contrast, Jurkat cells (Schneider *et al.*, 1977) behaved differently (Bröker *et al.*, 1993). The herpesviral transformed cell lines were strongly stimulated by cell-bound CD58, which is expressed on cells of various origin. This effect was mediated by CD2/CD58 interaction, and led to IL-2 production and enhanced proliferation (Mittrücker *et al.*, 1992). The functionality of CD3, CD4 and the IL-2 receptor was shown after stimulation by signal transduction parameters, by proliferation and by IFN-γ production (Bröker *et al.*, 1993; Weber *et al.*, 1993). The IL-2 dependent proliferation of transformed lymphocytes was strongly inhibited by soluble CD4 antibodies. This effect could be overcome by high doses of IL-2. In parallel, the activity and abundance of the CD4 bound fraction of the tyrosine kinase $p56^{lck}$ was diminished by the anti-CD4 treatment (Bröker *et al.*, 1994). A comparison of uninfected clones and their herpesvirus-transformed derivatives indicated that the growth transformation by herpesvirus saimiri is not based on a resistance to apoptosis (Kraft *et al.*, 1998). Herpesvirus saimiri transformed T cells can be driven into activation-dependent cell death after CD3 or TPA stimulation. This form of cell death is not mediated by Fas (CD95, Apo1)/Fas ligand interaction (Bröker *et al.*, 1997).

Characterized T-helper cell clones reacting specifically to myelin basic protein (Weber *et al.*, 1993; Meinl *et al.*, 1995b), tetanus toxoid (Bröker *et al.*, 1993), bovine 70 kDa heat shock protein (HSP70), *Lolium perenne* group I antigen, *Toxocara canis* excretory antigen, and to purified protein derivative from *Mycobacterium tuberculosis* (De Carli *et al.*, 1993) were successfully transformed and retained their HLA-restricted antigen specificity. The high basal proliferation activity, which is probably due to contact with CD58-bearing cells (Mittrücker *et al.*, 1992), may interfere with the demonstration of antigen specificity, since the antigen-presenting cells

alone cause a strong stimulation of the transformed T cells. This antigen-independent proliferation of the transformed antigen-specific clones could be reduced by using monoclonal antibodies against CD58 and CD2 together with HLA-transfected mouse L-cells as antigen-presenting cells (Weber *et al.*, 1993), or by starving the cells prior to antigen presentation (Bröker *et al.*, 1993). In all three cases, clear responses to antigen contact were noted, as measured by proliferation and cytokine production. IFN-γ production is the best readout parameter for activation (Bröker *et al.*, 1993; Weber *et al.*, 1993). Behrend *et al.* (1993) reported, that EBV-specific cytotoxic T lymphocytes (CTLs) retained their antigen-specific reactivity after viral transformation. In another study, a transformed tumour-specific CTL line recognized the autologous target only, if the tumour cells were pretreated with IFN-γ (Okada *et al.*, 1997).

Only a few specific cellular and biochemical features are changed in herpesvirus saimiri transformed T cells. The spontaneous growth is associated with CD2 hyperreactivity. Whereas primary T cells need the engagement of two epitopes on the CD2 molecule for activation, ligation of a single CD2 monoclonal antibody leads to the stimulation of herpesvirus-transformed T lymphocytes (Mittrücker *et al.*, 1992). IFN-γ secretion can be stimulated to very high levels (Bröker *et al.*, 1993; De Carli *et al.*, 1993; Weber *et al.*, 1993). The B cell specific tyrosine kinase Lyn is expressed in T cells transformed either by herpesvirus saimiri or by HTLV-1 (Yamanashi *et al.*, 1989; Wiese *et al.*, 1996; Fickenscher *et al.*, 1997).

When polyclonal T-cell populations were transformed by herpesvirus saimiri, T-cell cultures with TcR αβ are routinely obtained. These cultures are usually polyclonal or oligoclonal. There is no preference for expression of particular TCR Vβ genes. Several months after infection, a predominance of a reduced number of TCR Vβ gene families was found, which varied between parallel cultures. The most likely explanation is that the originally polyclonal T-cell cultures are overgrown by those clones that proliferate most rapidly. This is a general phenomenon in long-term cell culture that occurs in the absence of any specific stimulation (Fickenscher *et al.*, 1996a).

Various subgroup C virus strains are able to transform human T cells, to varying extents. Virus strain C484 transforms human T cells to a short-term, IL-2 independent growth (Medveczky *et al.*, 1993a). Different subgroup C strains (C488, C484, C139) were compared in the growth transformation of human cord blood T cells. The resulting clonal T-cell lines were either $CD4^+$ or $CD8^+$, and expressed either αβ or γδ TcRs. If transformed by the same virus strain, αβ and γδ clones were similar with respect to viral persistence, virus gene expression, proliferation, and Th1-type cytokine production. However, major differences were observed in T cells transformed by different subgroup C strains. Strain C139 persisted at low copy number, compared to the high copy number of prototype C488. The transformation-associated genes *stpC* and *tip* of strain C488 were strongly induced after T-cell stimulation. The homologous genes of strain C139 were only weakly expressed, and were not induced after activation. After CD2 ligation of a single CD2 epitope, C488 transformed T cells produced IL-2, whereas C139 transformed cells did not.

Correspondingly, C139 transformed T cells were less sensitive to cyclosporin A. IFN-γ production was induced to a similar extent in both C139 and C488 transformed T cells by the CD2 stimulus. Sequence comparison from different subgroup C strains revealed a variability of the *stpC/tip* promoter region and of the Lck-binding viral protein Tip. Thus, closely related subgroup C strains of herpesvirus saimiri cause major differences in the functional phenotype of growth-transformed human T cells (Fickenscher *et al.*, 1997).

So far, all the transformed T cells from normal donors that have been tested could be stimulated via TcR or CD3. Surprisingly, also cells with defects in TcR dependent signalling can be transformed by herpesvirus saimiri. The defect of the CD3γ chain did not prevent transformation (Rodriguez-Gallego *et al.*, 1996). The transformed T cells from a patient with atypical X-linked severe combined immunodeficiency showed a spontaneous partial reversion of the genetic defect affecting the IL-2Rγ chain (Stephan *et al.*, 1996). Fas-deficient T cells of a human SCID patient were efficiently transformed (Bröker *et al.*, 1997). Herpesvirus transformation seems promising for studying T cells from SCID patients.

The herpesvirus saimiri transformed human CD4 T cells provide a new productive lytical system for T lymphotropic viruses such as human herpesvirus type 6 (F. Neipel and B. Fleckenstein, unpublished observations) and HIV (Nick *et al.*, 1993; Saha *et al.*, 1996b; Vella *et al.*, 1997). The prototype viruses HIV-1_{IIIB} and HIV-2_{ROD} replicated rapidly in the cells, and caused cell death within 14 days. Also, a poorly replicating HIV-2 strain and primary clinical isolates grew to high titres. Herpesvirus saimiri transformed human CD4 T cells may be used for poorly growing HIV strains with narrowly restricted host cell range (Nick *et al.*, 1993). Moreover, herpesvirus transformed T cells can be persistently and productively infected with HIV. In comparison to conventional T-cell lines, the down-regulation of surface CD4 molecules is delayed (Vella *et al.*, 1997). Herpesvirus saimiri transformed $CD8^+$ T cells were shown to secrete a soluble HIV-inhibiting factor acting in a dependent manner on the NF-κB and NFAT-1 elements in the HIV LTR (Copeland *et al.*, 1995, 1996).

In recent years the use of herpesvirus saimiri has been focused on T cells from humans. The behaviour of this virus in various monkey systems is of importance because, on the one hand, herpesvirus saimiri is a tumour virus of New World monkeys, and, on the other hand, Old World monkeys such as macaques are the most used animal model for the close-to-human situation. In New World primate T cells, herpesvirus saimiri establishes a semipermissive infection. The cells do produce virus for long time periods, and are transformed (summarized in Fickenscher *et al.*, 1996b). Transcription of IL-2 and activity of IL-4 has been shown from such cultures (Chou *et al.*, 1995). Similarly to human T cells, the T lymphocytes from macaque monkeys can be growth transformed by herpesvirus saimiri (Akari *et al.*, 1996; Feldmann *et al.*, 1997; Meinl *et al.*, 1997). Some researchers have observed IL-2 dependence, while others have noted IL-2 independence. In many respects the transformed macaque T cells resemble their human counterparts. The phenotype of activated T

cells is preserved, and virus particles are not released. Antigen-specific T cell lines against myelin basic protein or streptolysin O retained their reactivity after transformation but, in contrast to their uninfected progenitor cells, the antigen specificity of the transformants was obscured by increasing concentrations of irradiated autologous blood cells as antigen-presenting cells. The MHC class II expressing transformed cells were able to present the antigen to each other in absence of autologous presenter cells (Meinl *et al.*, 1997). One major difference is the pronounced frequency of double-positive $CD4^+/CD8^+$ T cells, which are uncommon in humans. T cell immunology and T cell transformation from macaques is greatly hampered by reactivation of foamy virus, with which most rhesus monkeys in primate centres are infected (Feldmann *et al.*, 1997).

Retrovirus Transformed Human T Cells

HTLV-1 provided the first method for immortalizing human T lymphocytes. Like the EBV method for B cells, HTLV-1 has been used successfully for T cell transformation (Miyoshi *et al.*, 1981; Yamamoto *et al.*, 1982; Yoshida *et al.*, 1982; Popovic *et al.*, 1983). The resulting cell lines were valuable in terms of numerous scientific questions, which cannot be summarized in this article. However, the phenotype of HTLV-1 transformed human T cells needs to be discussed. In most cases the transformed T cells are $CD4^+/CD8^-$. However, $CD4^-/CD8^+$ human T cells can also be immortalized (Faller *et al.*, 1988; Persaud *et al.*, 1995). The transformed cells do release virus particles in most cases, and they express high amounts of CD25 (IL-2Rα chain) and HLA-DR molecules on their surface. In contrast to herpesvirus saimiri transformed T cells, HTLV-1 immortalized lymphocytes do not produce IL-2 (Grassmann *et al.*, 1994). Signal transduction studies have revealed that the B-cell tyrosine kinase Lyn is aberrantly expressed, which does normally not occur in T cells (Yamanashi *et al.*, 1989). This observation is similar in T cells transformed by herpesvirus saimiri (Wiese *et al.*, 1996; Fickenscher *et al.*, 1997). After a few months of culture, HTLV-1 transformed T cells tend to lose their antigen-specific reactivity, their TcR complex on the surface, their cytotoxic activity, and their dependence on exogenous IL-2 (Inatsuki *et al.*, 1989; Yssel *et al.*, 1989). This tendency reflects the development of autonomous growth in leukaemia development.

◆◆◆◆◆ VECTOR APPLICATIONS OF HERPESVIRUS SAIMIRI

Non-oncogenic herpesvirus saimiri strains have been used as eukaryotic expression vectors (Grassmann *et al.*, 1989). Recombinant viruses can infect and persist in human T cells and in a broad range of haematopoetic, mesenchymal and epithelial cells under selection conditions (Simmer *et al.*, 1991). The X region of HTLV-1 was inserted into herpesvirus saimiri

A-11 S4 (Grassmann *et al.*, 1989). This deletion variant of herpesvirus saimiri neither causes malignant disease in animals, nor transforms human lymphocytes in culture, since it lacks the terminal oncogene region (Desrosiers *et al.*, 1984, 1986). The HTLV-1 X region in the recombinant herpesvirus vector was able to transform primary human T lymphocytes to IL-2 dependent growth. These transformed lymphocytes are very similar to HTLV-1 transformed cells (Grassmann *et al.*, 1989). The *tax* recombinant transformed cells expressed lymphocyte activation genes (Kelly *et al.*, 1992), the CD4 surface molecule, MHC class II antigens, and the IL-2 receptor α-chain in large amounts. They contained episomes of the recombinant viruses in high copy number (Grassmann *et al.*, 1989). Deletion variants of the HTLV-1 X region were introduced into the viral vector in order to define the gene, which is responsible for transforming properties. The transcriptional activator Tax was found to be necessary and sufficient for lymphocyte immortalization in the context of the herpesviral vector (Grassmann *et al.*, 1992, 1994; Biesinger and Fleckenstein, 1992; Fickenscher *et al.*, 1993). The herpesvirus saimiri vector system (Alt *et al.*, 1991) may be useful for studying other transforming genes. It found further application in studying c-*fos* function (Alt and Grassmann, 1993). Overexpression of the proto-oncogene c-*fos* is known to induce transformation in various systems. The c-*fos* recombinant herpesviral vectors expressed large amounts of the oncoprotein upon persistent infection of human neonatal fibroblasts. However, these primary mesenchymal cells did not show any sign of transformation (Alt and Grassmann, 1993). Herpesvirus saimiri has been further used as a vector for growth hormone, for secreted alkaline phosphatase, and for green fluorescent protein (Desrosiers *et al.*, 1985b; Duboise *et al.*, 1996; Ensser *et al.*, 1998). The functional phenotype of herpesvirus saimiri transformed T cells suggests the use of herpesvirus saimiri as vector for human T lymphocytes. Reactivation of recombinant or wild-type virus from transformed human T cells has not been observed, but cannot be excluded. The techniques of homologous recombination and cosmid complementation could be applied for constructing replication-defective, but transformation-competent, deletion variants that preclude reactivation. Furthermore, additional genes could be introduced into subgroup C virus strains by these methods to express and to study those gene products in transformed lymphocytes. After further clarification of biosafety aspects, herpesvirus saimiri might be used as a gene vector for targeted amplification of functional human T cells in therapeutic applications.

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4 Generation and Characterization of Killer Cells

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INTRODUCTION

Many immunocytes have the capacity to kill other cells, or each other. In this chapter we concentrate on killer cells of the lymphocyte type. These fall into two main categories distinguished by their major histocompatibility complex (MHC) restriction or lack thereof, and their ability (and requirement) to be primed to the sensitizing antigen and to generate immunological memory therefor. MHC-restricted effectors are exclusively T lymphocytes and are commonly known as cytotoxic T lymphocytes (CTLs). They usually recognize antigen in the context of self MHC class I molecules and constitute the major category of antiviral effector cells. They are almost always $CD8^+$ due to the nature of the MHC molecule recognized. In contrast, $CD4^+$ T lymphocytes recognize antigen in the context of MHC class II molecules and may sometimes be cytotoxic to the cells that they recognize. 'Natural killing' is the term given to the

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phenomenon of MHC-unrestricted cytotoxicity mediated by a variety of lymphoid cell types without prior sensitization and without the generation of immunological memory. Natural killer (NK) cells are also involved as a first line of defence in protection against viral infection. The majority of NK cells can be distinguished from T cells by their surface markers, but TCR1 (γ/δ) T cells form an important group also capable of mediating NK-like activity. In this chapter we limit the discussion to the generation and characterization of $CD8^+$ 'classical' CTLs and the $CD16^+$ $CD56^+$ 'classical' NK cells in the human.

◆◆◆◆◆◆ NATURAL KILLER CELLS

Phenotyping and Isolation of Natural Killer Cells

Phenotyping

Several surface markers are useful in distinguishing NK cells from other cell types, although many are shared with T cells (e.g. CD2, CD7, CD8, CD43, CD122). NK cells also express members of the β_2-integrin family shared with myelomonocytic cells (e.g. CD11b/CD18). However, other differentiation molecules show a restricted expression on NK cells when compared to other lymphocytes. Thus, NK cells express CD56 (N-CAM), CD16 (the FcγRIII) and CD94 (Kp43), NKRP1 or NKG2 gene products. Human NK cells are CD3ε negative, but the ζ chain, which in T cells are associated with the TCR, is coupled to the CD16 receptor in NK cells. A family of clonally expressed NK inhibitory receptors is also predominantly expressed in NK cells. These recently defined markers will be useful for characterizing heterogeneous NK cell populations (Moretta *et al.*, 1997). These receptors, designated KIRs (killer inhibitory receptors), belong to the immunoglobulin superfamily and have either 2 (p58) or 3 (p70) domains, and specifically interact with HLA class I molecules. The anti-p58 (CD158a and 158b) monoclonal antibodies (mAbs) GL183, EB6 and HP3E4 bind receptors that interact with human leukocyte antigen (HLA)-C antigens, whereas p70 (NKB1) appears to be the NK receptor for the HLA-Bw4 public determinant present on the HLA-B*5101, -B*5801 and -B*2705 antigens. The use of NK clones has defined three NK specificities according to their capacity to recognize class I polymorphic motifs. Namely, NK1, defined by NK clones inhibited by HLA-C alleles with Lys80; NK2, by those HLA-C alleles with Ser77 and NK3, by NK clones inhibited by HLA-Bw4 alleles with Ile80. Another family of inhibitory receptors is represented by molecules of the C-type lectin superfamily, namely dimers composed of the CD94 and CD94 associated protein (CD94AP), which can also recognize HLA class I antigens (HLA-B and HLA-G antigens) inhibiting NK cytotoxicity. The p58 and p70 inhibitory receptors possess immunoreceptor tyrosine based inhibitory motifs (V/LXXYXXL) (ITIMs) in their intracellular tails. Recent results indicate that CD94 associated proteins correspond to the

NKG2A encoded molecules, which also have ITIMs in the intracytoplasmic domain.

Because of their KIRs, NK cells are only effective against HLA-allele-negative cell lines (Garrido *et al.*, 1997). Non-transfected and HLA-allele-transfected C1R and 721.221 cell lines can be used to analyse HLA-induced inhibition of NK cytotoxicity. When cytotoxicity against an HLA class I transfectant is diminished by 50% or more compared with the untransfected parental cell line, it can be concluded that that particular NK cell clone is able to recognize the HLA class I molecules expressed by the transfectant. Alternatively, NK cells can be directly typed for expression of KIRs using mAbs as described in the following protocols. Other surface molecules also expressed in human NK cells or NK subpopulations and useful for their definition in health and disease are: CD26, CD27, CD29, CD45, CD57, CD69, CD81, p38 (C1.7.1) and PEN5. There are several standard methods for surface marker phenotyping, and these are described below.

Membrane immunofluorescence staining

mAbs are commonly used to identify surface antigens on viable cell suspensions by either: (i) direct immunofluorescence, which employs specific antibodies conjugated with fluorochromes; or (ii) indirect immunofluorescence, which uses a two-step procedure, where the specific antibody is unlabelled and is tagged by a conjugated anti-Ig antibody.

- 2×10^5 peripheral blood mononuclear cells (PBMCs) are incubated with each labelled mAb in V-bottom plates for 30 min at 4°C, washed twice with phosphate buffered saline (PBS) plus 2% fetal calf serum (FCS) and 0.1% sodium azide (PBS-FCS-Az) and resuspended in 1% paraformaldehyde for the analysis. Mixtures of two or more differently labelled mAbs can be incubated simultaneously with the same cells for muilti-colour fluorescence. Negative control cells should be incubated with Ig isotype control mAb.
- 2×10^5 PBMCs are incubated as for the direct method, but unlabelled antibodies are used. After washing and resuspension, cells are incubated again with labelled goat anti-mouse immunoglobulin (Ig) for 30 min at 4°C. After final washing, the cells are resuspended in 1% paraformaldehyde for the analysis. Negative control cells are incubated only with the secondary labelled antibody.

Intracellular antigens

This method is useful for evaluating the presence of intracellular granule content, in particular perforins in NK cells (Mariani *et al.*, 1996).

PBMCs (10^6) are resuspended in 1 ml cold 2% paraformaldehyde solution in PBS, incubated for 1 h at 4°C in the dark, and then centrifuged for 10 min at 250*g* and 4°C. The fixed cells are resuspended in 1 ml 0.2% Tween 20 in PBS at room temperature and incubated for 15 min at 37°C. PBS-FCS-Az (3 ml) is added and the suspension is centrifuged for 10 min at 250*g* at room temperature. The cell pellet is then incubated with an optimal dilution of an anti-human perforin mAb for 30 min at 4°C; positive cells are developed using a 1:20 diluted goat anti-mouse Ig conjugated with FITC for 30 min 4°C. The cell pellet is washed with PBS-FCS-Az and analysed.

Flow cytometry

Optical/electronic instruments that measure cell size and phenotype by the presence of bound fluorochrome-labelled antibodies are now widely used in place of a manual fluorescence microscopy for the analysis of cells labelled as described above. Several models are commercially available in two forms: analytical only, and analytical and sorting machines ('cell sorter').

A single-cell suspension labelled with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugated antibodies is forced through the nozzle of the machine under pressure. The cells confined to the axis of the fluid stream by a concentric sheath of cell-free fluid pass through a laser beam focused on the stream. The light scattered and reflected by the cells and emitted by excited fluorochromes bound to the cell membrane is collected by a suitable arrangement of lenses, optical filters and photo-electric devices. The electrical signals are analysed, processed and stored by a computer. When the machine is used to separate subpopulations of cells, these electrical signals are also used to activate the cell-sorting process. The present protocol was developed on the FACStar Plus machine (Becton Dickinson), equipped with a 4-W argon ion laser (Spectra Physics), operating at a wavelength of 488 nm in order to excite both FITC and PE fluorochromes. Forward light scatter or 'forward scatter' (FLS) (2–15°) was collected with a 0.5% neutral density filter in front of the photodiode. This parameter is a measure of cell size and is useful for discriminating between viable and dead cells and between nucleated cells and erythrocytes. Perpendicular light scatter or 'side scatter' (PLS) (75–105°), was measured collecting 5% of the light through a 488 nm bandpass filter with a photomultiplier tube (PMT). This is an indicator of the heterogeneity of cell structure ('granularity'). The simultaneous measurement of FLS and PLS allows the identification of different subpopulations of white blood cells. FITC green fluorescence was selected using a 515-nm low-pass filter and a 530 ± 10 nm bandpass filter. PE red fluorescence was selected by a 575 ± 15 nm bandpassfilter. The PMT for the detection of green and red fluorescences was operating at 500 V.

A quantity of 10^5 labelled cells should be analysed for each sample. In order to apply optimal gating for the identification and exclusion of monocytes from lymphocyte populations, mononuclear cells are analysed on a scattergram using rectangular or polygonal computer-generated windows. The number of lymphocytes contained either in the regular or in the polygonal windows should be the same, but the number of contaminating monocytes can be decreased by two-fold in the polygonal window. In addition, care must be exercised not to use a too-restricted lymphocyte gate in an attempt to achieve lower monocyte contamination. This is because this can lead to a loss of lymphoid cells which does not appear to be random, but seems to affect mainly the CD16 population with NK activity. This point should be borne in mind when setting gates for analysing NK cells in whole PBMCs. These cells produce forward and perpendicular scatter signals that are higher than for other lymphocyte subsets, and therefore are mainly located in the area of the scattergram that divides lymphocytes from monocytes. These data are in accordance with the large granular lymphocyte morphology of NK cells.

Therefore, the use of the polygonal windows seems to be beneficial to reduce monocyte contamination without selective loss of NK lymphocytes, and may be particularly helpful in the analysis of pathological samples.

Isolation

The relatively small amount of NK cells within the blood has made it difficult to obtain these cells in pure form and in large enough numbers to perform functional tests. Cytotoxic NK cells can be purified by cell sorting, immunomagnetic purification or complement-dependent depletion of other cell types. In the past NK cells have been isolated based on their physical properties ('large granular lymphocytes'), but this method has now been superceded. Because NK cells can be kept functionally active in culture for several days with interleukin-2 (IL-2), a new simple procedure for obtaining NK cells is to isolate mononuclear cells from blood, remove the TCR^+ T cells with anti-CD3 antibody, and culture the cells for several days with IL-2 and Epstein–Barr virus (EBV) transformed feeder cell lines. These cells are all $CD3^-$, but are positive for NK markers. NK cells can also be propagated with IL-2 for prolonged periods of time as lines or clones by using appropriate stimulator or feeder cells (Luque *et al.*, 1996).

Cell sorting

PBMCs are incubated with different fluorochrome-labelled mAbs specific for the cell to be purified. If the sorted cells are to be used in functional studies, mAbs that do not activate the cells must be used. To sort the cells according to their detected characteristics, the fluid stream through the flow cytometer is induced to break up into separate droplets by a microscopically small vertical vibration of the nozzle assembly. At the end of the unbroken stream, the droplets are electrically charged by applying a charging

pulse to the whole stream. The polarity of this pulse is predetermined by the experimenter. The charged drops pass through a transverse electric field formed between two metal plates differing in potential. Positively and negatively charged drops are deflected towards the corresponding opposite plates. The resultant separate droplets are collected in different left and right tubes, and the central component of the stream is discarded.

Preparation of human natural killer cells by immunomagnetic separation

This technique also allows for a positive and a negative system of selection. Both positive and negative selection with magnetic beads may be accomplished by indirect or direct antibody-labelling methods. With the indirect method, the cells are incubated with mouse mAbs that subsequently adhere to anti-mouse IgG-coated beads. With the direct method the cells are directly incubated with beads coated with mAb. In the following protocol, human NK cells are purified by negative selection, using an indirect method.

Take 50×10^6 PBMCs at 5×10^6 PBMC ml^{-1} in complete medium (CM: RPMI 1640 with *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES) buffer supplemented with 10% FCS, antibiotics and 2 mM L-glutamine) and incubate in a Petri dish for 60–90 min at 37°C in 5% CO_2, for monocyte depletion. Non adherent cells are recovered and washed twice with PBS and diluted in 2 ml of medium. Incubate the cells in prewashed nylon wool for 45–60 min at 37°C in 5% CO_2, for B-cell depletion. The non-adherent (T/NK cells) are recovered by washing the column with 20 ml CM, resuspended in 500 ml and incubated with anti-CD3 mAb (10–25 μg) (anti-Leu 4, Becton-Dickinson) for 30 min at 4°C. Goat anti-mouse (GAM) coupled magnetic beads (0.7×10^8 to 0.9×10^8) (Dynal, Oslo, Norway) are now added for 30 min at 4°C under gentle shaking for T-cell depletion. Add 9 ml PBS and use a magnetic particle concentrator (MPC1, Dynal) to remove the cells rosetting with the beads. Collect the supernatant containing $CD3^-$ cells. Alternatively, whole PBMCs ($5\times10^6 ml^{-1}$) can be incubated directly with CD3, CD4, CD19 and CD14 mAb at 10 μg ml^{-1} for 30 min at 4°C, and then with GAM beads for 45 min at 4°C. Cells and beads should be gently mixed throughout. After the incubation with beads, a magnet is used to separate beads with attached CD3/CD4 T cells, CD14 monocytes and CD19 B cells from NK cells. Free cells are recovered, centrifuged and washed with complete medium. The cell population obtained should be 75–95% $CD56^+$ and $CD16^+$, <10% $CD3^+$ and <5% $CD14^+$ and $CD19^+$, as has been routinely established by flow cytometry.

Separation of natural killer cells by complement-mediated lysis

This is a classical, but frequently employed, technique used to obtain subpopulations of lymphocytes by negative selection, but is limited to complement-fixing mAbs.

The cells (5×10^6 to 1×10^7 ml^{-1}) are resuspended in medium containing the optimal dilution of antibody. Cells are maintained at 4°C to prevent capping. Sodium azide (10 mM) may also be used for the same purpose but, because of its interference with metabolism, it must not be used when the cells are purified for functional studies. After incubation for 30 min at 4°C with periodical shaking, the cells are pelleted, washed with cold medium and resuspended in the original volume of warm medium containing complement at the optimal dilution. The suspension is incubated for 45 min at 37°C, with regular shaking. At the end of incubation time, the cells are centrifuged, washed twice with complete medium containing 5% serum or 0.2% bovine serum albumin (BSA) and tested for viability, purity and recovery.

Activation of natural killer cells with interleukin-2

The cells obtained as indicated above can be cultured in 5% CO_2 at 37°C for 7–10 days in the presence of 500 U ml^{-1} IL-2, without further activation requirements. However, for better efficiency of NK expansion, the addition of irradiated feeder cells is recommended. EBV-transformed cell lines, in particular the RPMI 8226 cell line, are commonly used as feeder cells for NK cell activation.

Generation of natural killer cell clones

Isolated NK cells obtained as indicated above are incubated with feeder cells (10^5 NK cells with 5×10^4 irradiated (40 Gy) autologous PBMCs and irradiated (100 Gy) allogeneic EBV-transformed cells (1 : 1 mixture) in a total volume of 2 ml CM containing 250 U ml^{-1} IL-2) in a 24-well cluster plate. Incubate the plate for 4 days in a humidified 37°C, 5% CO_2 incubator. Remove 1 ml medium and replace with 1 ml containing 500 U rIL-2 (final concentration 250 U ml^{-1}). Incubate the plate for 3 days. Wash cells by centrifuging for 5–10 min at 1500 rpm and count them. Place cells (at the limiting dilution) into each well of a 96-well round-bottomed microtitre plate containing 10^5 irradiated autologous mononuclear cells, 5×10^4 irradiated allogeneic EBV-transformed cells and 250 U ml^{-1} rIL-2 in a final volume of 200 µl. Incubate plates at 37°C, 5% CO_2. Remove 100 µl medium and replace with 100 µl complete medium with 500 U ml^{-1} rIL-2. Incubate for 3 more days. Remove 100 µl medium. Add 5×10^4 irradiated allogeneic EBV-transformed cells in CM and 500 U ml^{-1} rIL-2. Incubate for another 4 days. Remove 100 µl medium and again replace with 100 µl CM containing 500 U ml^{-1} rIL-2, and incubate for a further 3 days. Identify growing wells by visual microscopy. Wash cells in serum-free RPMI, resuspend at 10^6 cells ml^{-1} and place 2 ml of this suspension in each well of a 24-well microtitre plate. (At this point some cells can be cryopreserved and the remainder cultured under different conditions to establish optimal parameters for each particular clone.) After 2–3 days, remove 1 ml and add fresh medium containing rIL-2 only. Incubate for 4–7 days. Control growth and feed the cells with fresh medium containing rIL-2 weekly, depending of the growth rate.

◆◆◆◆◆ CYTOTOXIC T CELLS

Generation of cytotoxic T lymphocytes

One problem hindering the successful and reproducible generation of $CD8^+$ CTLs is the common finding that the *in vitro* sensitization and culture techniques routinely employed in cellular immunology strongly favour the outgrowth of $CD4^+$ helper cells (although many of these, particularly 'T-helper 1 (Th1) like' cells also possess some cytotoxic potential). To obtain a high yield of antigen-specific MHC class I-restricted $CD8^+$ cells it is necessary to enrich for CD8 cells or deplete for CD4 cells at the beginning. This can be done using antibody-based techniques involving separation with magnetic beads, FACS or solid-phase absorption. When generating antigen-specific cells it is also of critical importance to use high-efficiency antigen-presenting cells (APCs). Activated B-cell blasts or dendritic cells are currently favoured. Space considerations do not allow protocols for all these techniques to be given here; instead we present a hybrid technique that has been used successfully for the generation of CTLs to antigenic peptides *in vitro* (Van Elsas *et al.*, 1996).

Preparation of antigen-presenting cells

The first step in the procedure is to prepare the APCs and the responders, starting from normal human PBMCs. The PBMCs are plated in six-well plates at 20×10^6 per well in 3 ml RPMI + 10% FCS, incubated for 2 h at 37°C, gently swirled, washed, and then the medium containing non-adherent cells is collected. The latter cells are cryopreserved for later use as responders. To prepare the APCs, add 2.5 ml RPMI + 10% FCS containing 800 U ml^{-1} of GM-CSF (8×10^8 U mg^{-1}) and 500 U ml^{-1} of IL 4 (10^7 U mg^{-1}) to each well with adherent cells and culture for 2 days at 37°C. Then add 2.5 ml fresh RPMI + 10% FCS containing 1600 U ml^{-1} granulocyte–macrophage colony stimulating factor (GM-CSF) and 1000 U ml^{-1} IL-4, and 2 days later remove 2.5 ml culture medium from each well and replace by 2.5 ml fresh RPMI + 10% FCS containing 1600 U ml^{-1} GM-CSF and 1000 U ml^{-1} IL-4. Next day, remove 1 ml culture medium from each well and replace with 1 ml fresh medium containing 750 U ml^{-1} of γ-interferon (IFN-γ) (10^7 U mg^{-1}) and 250 U ml^{-1} IL-1α (10^8 U mg^{-1}) (final concentrations 150 and 50 U ml^{-1}, respectively). One day later, harvest the cultured APCs and wash twice with RPMI + 1% human serum (HS). Use tubes 'precoated' with RPMI + 5% HS for these cells, because they easily adhere to plastic. Resuspend the cells in 1 ml RPMI + 1% HS containing 50 μg ml^{-1} of the desired peptide antigen (e.g. viral epitope) and 3 μg ml^{-1} β_2-microglobulin (β_2M). Incubate at 37°C for 4 h; gently resuspend every hour. Finally, irradiate at 25 Gy and wash twice with RPMI + 1% HS, resuspend at 0.3×10^6 ml^{-1} in RPMI + 5% HS.

Responder cells

Thaw the non-adherent fraction (those previously frozen); keep 25% of these cells and deplete the remaining 75% of $CD4^+$ cells as follows: Wash cells three times with cold RPMI + 1% FCS, add a three- to four-fold excess of CD4 Dynal beads and incubate at 4°C for 30–45 min, wash cells three times with cold RPMI + 1% FCS while using a magnetic stirrer. Collect the supernatant. Count the resulting CD4-depleted population. Reconstitute the responders with a small proportion of CD4 cells. To do this, consider the non-depleted cells as 40% $CD4^+$. Add non-depleted cells to the CD4-depleted population to give a final dose of about 10% $CD4^+$ cells (i.e. if total CD4-depleted cells = x, add $0.33x$ of non-depleted cells).

Initiation of cytotoxic-T-lymphocyte stimulation cultures

Use at least 15×10^6 responders prepared as above (and therefore 1.5×10^6 stimulator cells (i.e. APCs) to give a responder/stimulator ratio of 10:1), per induction culture. Resuspend these responders at $3 \times 10^6 ml^{-1}$ in RPMI + 5% HS containing 20 ng ml^{-1} of IL-7 (2×10^8 U mg^{-1}) and 100 pg ml^{-1} of IL-12 ($2 \times 10^5 U/mg^{-1}$) and plate in 24-well plates (1 ml per well). Add 10% stimulator cells (i.e. 0.3×10^6 in 1 ml per well). Seven days later, remove 1 ml medium and replace with 1 ml RPMI + 5% HS containing 20 ng ml^{-1} of IL-7. After a further 5 days, harvest the sensitized cells, separate over Ficoll/Hypaque, wash once and count the viable cells. Resuspend at $1.5 \times 10^6 ml^{-1}$ in RPMI + 5% HS and keep the tube at 37°C. Thaw 4×10^6 autologous PBMCs per 1.5×10^6 responder cells. Use serum-free RPMI for thawing, wash once in serum-free RPMI and irradiate at 60 Gy. Wash again in serum-free RPMI and resuspend at $4 \times 10^6 ml^{-1}$ in RPMI + 5% HS. Plate in 24-well plates (1 ml per well) and incubate for 2 h at 37°C. Swirl the plates gently and collect medium containing non-adherent cells. Gently wash cells with 2 ml RPMI + 5% HS to remove remaining non-adherent cells and add 0.5 ml of RPMI + 5% HS containing 20 μg ml^{-1} of peptide and 3 μg ml^{-1} of beta-2-microglobulin (β_2M) to the adherent cells and incubate for 2 h at 37°C. After this, remove medium, gently wash once and add 1 ml of responder cell suspension (i.e. $1.5 \times 10^6 ml^{-1}$). After a total of 2 weeks, add 1 ml RPMI + 5% HS containing 20 IU ml^{-1} of IL-2 (1.8×10^7 U mg^{-1}) to each well. After a further 5 days, restimulate as before. Whether isolation of live cells over Ficoll/Hypaque is necessary will depend on the quality of the cultures and the total number of cells. After a total of 3 weeks, add fresh medium + IL-2 as after 2 weeks, and 5 days later restimulate again. Check the CD4/CD8 ratio of responder cells at this time. If $CD4^+$ cells are found to predominate in the cultures, CD4-depletion must be performed again, as at the beginning of the procedure. After 4 weeks, feed with fresh medium + IL-2, and harvest responder cells 5 days later. Whether isolation of live cells over Ficoll/Hypaque is necessary will again depend on the quality of the cultures and the total number of cells. The effector cells are now ready for testing in a cytotoxicity assay. At this point, the effector cells may also be subjected to cloning.

Cloning cytotoxic T lymphocytes

Once populations of sensitized CTLs have been obtained, they can be successfully cloned by limiting dilution under standard conditions (Pawelec, 1993):

1. Dilute the cells for cloning in the culture medium in which they were sensitized, but to which 20 U ml^{-1} of IL-2 is added. Set the concentration so that 10 µl contains 45, 4.5 or 0.45 cells. Then plate 10 µl of the 0.45 suspension to 60 1-mm diameter wells of culture trays ('Terasaki plates') and leave in a vibration-free area for 1 h. Check the distribution of cells in the wells visually by using an inverted microscope, taking care to look around the edges of the wells. According to the Poisson distribution, only a maximum of 37% of the wells should contain cells. Readjust dilutions if necessary, and recheck.
2. Plate at least five trays with the 0.45 cells per 10-µl suspension, one with 4.5 and one with 45 cells per 10 µl, and add a constant number of feeder cells to each well. Irradiated PBMCs are commonly used as feeder cells at 1×10^4/well. Use autologous PBMCs, a mixture of autologous PBMCs and autologous B-lymphoblastoid line cells, or other appropriate APCs, in the presence of specific antigen. Alternatively, use an antigen-non-specific stimulus such as 50 ng ml^{-1} of the anti-CD3 monoclonal antibody OKT3, or 2 µg ml^{-1} of the mitogen phytohaemagglutinin (PHA), together with the same number of allogeneic or autologous PBMCs.
3. Stack plates and wrap in aluminium foil for ease of handling and as a precaution against contamination. Incubate for about a week and then examine the plates using an inverted microscope. Transfer the contents of positive wells (more than one-third full) to 7-mm diameter flat-bottomed microtitre plate wells with fresh medium and 1×10^5 of the same feeder cells as before. Check Terasaki plates again at intervals of a few days up to 2–3 weeks of age, in order to identify late developers, and transfer these also. Check the microtitre plates every few days, and identify wells that are becoming crowded within a week after transfer. These must be split 1:1 into new culture wells and re-fed with medium (but not feeder cells). After 1 week in microtitre plates transfer the contents of wells with growing cells to 16-mm diameter cluster plate wells with 2×10^5 to 5×10^5 of the same feeder cells, and fresh medium. Observe after 3–4 days and establish which wells are already full or nearly full. The former should be divided into four, the latter into two, with fresh media, but no more feeders. After a total of one week in cluster plates, count the number of cells in each clone and split to 2×10^5 per well, again with 2×10^5 to 5×10^5 feeders per well and fresh medium. Feed after 3–4 days with fresh medium, and split again if necessary. Clones successfully propagated in cluster plate wells for this second week are taken to be established. At this point, some (or all) can be

cryopreserved and the remainder cultured under different conditions, in order to establish optimal parameters for each particular clone. Having a frozen stock enables one to test different culture conditions in order to optimize growth, without the danger of losing the whole clone.

4. Test whether established clones can be propagated with the most convenient feeder cells (80 Gy irradiated B-lymphoblastoid cell lines) instead of PBMC feeders. Most T-cell clones (TCCs) flourish on B-lymphoblastoid cell line (B-LCL) alone, but some appear (for unknown reasons) to benefit from the presence of PBMCs as well (this is especially true during cloning). Propagation of the TCCs on PBMC feeders can of course also be continued, but many workers may find it easier to grow large amounts of B-LCL than to isolate the PBMCs. Furthermore, PBMCs from the autologous donor may not be freely available in sufficient amounts for large-scale propagation of numerous clones. The international availability of well-characterized MHC homozygous B-LCL makes it possible to match the feeder cell to the specificity of the TCCs being propagated, and to enhance the antigen-presentation function of the feeders.
5. As a matter of convenience, it is easier to grow TCCs in scaled-up culture vessels than in cluster plates, but not all clones can be adapted to growth in flasks. This must also be tested for each clone, using 1×10^5 and $5\times10^5 ml^{-1}$ TCCs with an equal number of feeders in tissue culture flasks. Those clones not growing under these conditions can (rarely) be adapted to flask growth by altering the amounts or concentrations of TCCs or feeders seeded, or by increasing or decreasing the frequency of stimulation and/or feeding. It is unknown why some TCCs fail to flourish in flasks.
6. Establish restimulation parameters for each clone. T cells require periodic reactivation through the T-cell antigen receptor in order to retain responsiveness to growth factors. This can be accomplished specifically or non-specifically. All clones can be propagated with weekly restimulation; some but not all can be propagated with restimulation only every 2 weeks. Human T-cell clones can be easily cryopreserved using the same protocols that are suitable for freezing resting T cells. Clones developing with different kinetics can thus be collected and conveniently tested for cytotoxicity in the same experiment.

◆◆◆◆◆ CYTOTOXIC ASSAYS FOR NATURAL KILLER CELLS AND CYTOTOXIC T LYMPHOCYTES

Effector Cells

Effector cells generated as described in the preceding sections (CTL, NK cells, clones or lines) are suspended in RPMI 1640 medium containing 10% FCS. The cell concentration required will depend on the nature of the effector population and its level of cytotoxic activity. A

starting effector/target ratio of 50:1 or 100:1 will be required for assaying freshly isolated NK cells, whereas for highly active cloned effectors this ratio may be at most 10:1. In addition to NK and CTL assays, antibody-dependent cellular cytotoxicity (ADCC) measures redirected lysis of either type of effector, provided that an antibody specific for a target antigen can bind the target cell and also bind and activate the effector cell.

Target Cells

Commonly used target cells for measuring resting and activated NK lysis are K562 and DAUDI lines, respectively. Other cell lines used as targets are C1R or 721.221 EBV transformed cell lines, which do not express HLA class I antigens on the surface. A variety of NK-resistant cell lines can be used to analyse ADCC by using NK cells and IgG antibodies specific for the target cells. The P815 murine mastocytoma cell line is also resistant to lysis by resting human NK cells and is frequently used to analyse redirected lysis (e.g. lysis of the target in the presence of antibodies against NK-triggering structures). Convenient targets for measuring allospecific or antigen-specific lysis are B-lymphoblastoid cell lines, which are easy to grow, express high density MHC molecules and can take up and process antigens. Peptide-loaded target cells (e.g. B-LCL) are used to determine the peptide specificity of effector populations. Note that, after initial testing of the peptide specificity, additional experiments are needed in which one tests the recognition of targets that present limiting amounts of the peptide of choice at their surface (titration of the peptide through testing of 10-fold dilutions). Only CTLs that are highly sensitive in that they recognize peptides in the 10–100 pg ml^{-1} range are to be considered high-affinity CTLs that are likely to react with physiological amounts of naturally processed antigen. In the case of peptide-primed CTLs, target cells that derive the relevant immunogenic peptide through processing of endogenous proteins are, of course, also required. They are needed to determine whether the CTLs obtained not only recognize exogenously loaded peptide, but also naturally processed antigen.

PHA or concanavalin A (ConA) blasts can also be used as targets for allospecific CTL. For the generation of target PHA or ConA lymphoblasts, PBMCs at 2×10^6 cells ml^{-1} are cultured for 2–3 days in RPMI 1640 with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate and 1 μg ml^{-1} of PHA or 5 μg ml^{-1} of ConA. Before use, dead cells are removed by Lymphoprep separation.

Most other types of large cell (e.g. tumour cells or fibroblasts) are also suitable for use in cytotoxicity assays. However, for the chromium- or calcein-release assays described below, small resting cells are unsuitable because they do not take up sufficient label into their cytoplasm.

To label the selected target cells, about 1×10^6 cells are incubated together with 3.7×10^6 Bq (= 100 μCi) of sodium^{51} chromate in about 0.25 ml complete medium (behind lead shielding). The specific activity of the chromium should be around 2.22×10^{10} Bq (600 mCi) per mg chromium (used no later than 15 days after the reference day). After 90 min at 37°C, during which time the cells are shaken every 15 min, the cells are washed three times in cold RPMI + 10% FCS, the last wash being performed just before seeding the cells. Labelled target cells are counted and adjusted to an appropriate concentration for distribution into the wells of U-form or V-form microtitre plates. It is generally sufficient to use 5×10^3 labelled target cells per well, which can be added in 100 μl of complete medium. During all procedures after incubation with chrome, the target cells should be kept on ice in order to reduce spontaneous isotope release before the test.

Having plated the appropriate number of wells with target cells, 100-μl aliquots of titrated numbers of effector cells are added in doubling or tripling dilutions in triplicate to give at least four different effector/target ratios. Target cells must also be incubated in medium alone in order to measure spontaneous release of isotope, and in lysis buffer (e.g. 1% Triton X-100 detergent) to measure the maximum release possible. For most assays, incubating the plates at 37°C for 4 h is sufficient for measurement of specific lysis. At the beginning and at the end of the incubation period, the plates are centrifuged at 4°C for 7 min. After this, or for some assays after an extended time, 100 μl cell-free supernatant is carefully removed with a pipette and transferred to a tube or other receptacle for measuring released radioactivity in a γ counter. Alternatively, the radioactivity in approximately 85% of the supernatant can be collected in filters with the Titertek microplate semi-automatic harvesting system, which uses cellulose acetate absorption cartridges (Skatron, Lyerbyen, Norway). The percentage specific chromium release (= percentage lysis) is then calculated according to the simple formula:

$$\%\ \text{Lysis} = \frac{\text{Experimental CPM} - \text{Spontaneous CPM}}{\text{Maximum CPM} - \text{Spontaneous CPM}} \times 100$$

We consider sufficient labelling to be at least 1 cpm per cell, measurable in the maximum release assay. An optimal spontaneous release should be less than 10% of the maximum, but up to 33% is commonly accepted, especially for 'difficult' targets such as fresh leukaemia cells.

Calcein release assay

Because the chromium release assay involves unavoidable exposure of the experimenter to γ-irradiation, attempts have been made repeatedly to establish non-radioactive cytotoxicity assays. Moreover, the chromium release assay suffers from the disadvantage that the spontaneous release may be very high, particularly with extended assays, and with difficult target cells (e.g. chronic myelogenous leukaemia blasts). We describe here the alternative assay (CARE-LASS) with which we have had the most favourable experience. In this case, the target cells are not loaded with radioactive chromium but with a fluorescent dye, which is released into the supernatant on lysis (Lichtenfels *et al.*, 1994). Otherwise, the entire procedure is analogous to the chromium release assay, but requires less time to perform.

Cells to be labelled are washed, resuspended and incubated for 30 min at 37°C in a final concentration of 25 μM calcein–AM (provided by Molecular Probes Inc. at 2.5 mM in dimethylsulfoxide (DMSO)). The cells are then washed twice, counted and resuspended at an appropriate concentration. Effector and target cells are then plated as in the chromium-release assay, but also including target cells in medium alone (spontaneous release) and in lysis buffer, which can be Triton X-100 for this technique as well (maximum release). Incubate for 2–4 h and then pipette cell-free supernatant into the corresponding wells of a new microtitre plate. The calcein fluorescence of each well can now be measured in an automated plate-reading fluorometer with an excitation filter setting of 485/20 and an emission filter setting of 530/25 (e.g. the Fluoroskan Ascent from Labsystems Oy, Helsinki).

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5 Measuring Human Cytokine Responses

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INTRODUCTION

The term 'cytokine' has been used to describe a diverse group of low molecular weight (generally <20 kDa) protein mediators, which have a broad spectrum of immunoregulatory effects and which are produced by a variety of cell types. Although many cytokines were originally defined and named after the particular biological functions that they display, the development of recombinant DNA technology has permitted the classification of a plethora of factors and activities into a growing list of well-defined proteins. From a functional point of view, cytokines have several features in common.

- Most, if not all, cytokines have redundant activities, as reflected by their ability to perform similar functions. Examples are interleukin (IL) 2, IL-4, IL-7, IL-12, IL-15 and even IL-10, which all have T-cell growth promoting activities, and IL-4 and IL-13, which share most of their functional activities, such as induction of B-cell proliferation and differentiation, induction of immunoglobulin (Ig) G4 and IgE isotype switching, induction of mast cell differentiation and downregulation of secretion of proinflammatory cytokines by macrophages.

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- Their effects are pleiotropic, affecting many different target cells, which is reflected by the functional expression of receptors for many cytokines, such as IL-1, IL-2, IL-4, IL-6 or IL-10 on most cells of the immune system.
- Their secretion is highly regulated, because of the potential for tissue destruction, and they are often secreted during bursts of immune responses.
- Cytokines interact with specific high-affinity cell surface receptors, many of which have been cloned, which is followed by a cascade of signal transduction events, resulting in mRNA synthesis and protein secretion. For a large number of ILs these signal transduction events have been shown to be associated with the phosphorylation of specific tyrosine kinases, members of the Jak-Stat signal transduction system (reviewed in Ihle, 1995). In addition, most cytokines are glycosylated, giving rise to considerable molecular heterogeneity, and the different sugar molecules are thought to be important for receptor binding and modulation of receptor-mediated signal transduction.
- Many cytokines are able to induce or inhibit each other's synthesis, including their own, resulting in the creation of regulatory networks. An example of reciprocal inducing activity of cytokines, resulting in a positive feedback is given by the action IL-12 and interferon-γ (IFN-γ). The secretion of IL-12 by activated monocytes strongly induces the production of IFN-γ by T cells and natural killer (NK) cells, which in turn enhances the production of IL-12. The inhibitory action on cytokine production is exemplified by IL-4, IL-10 and IL-13, which efficiently block the secretion of proinflammatory cytokines, such as IL-1, IL-6 and tumour necrosis factor α (TNFα), and enhance the production of IL-1 receptor antagonist (IL-1RA) by activated macrophages, whereas IL-10, which has relatively late production kinetics, is able to inhibit its own synthesis via an autocrine feedback mechanism.
- Mixtures of cytokines often have synergistic effects, as compared to the effect of each of them separately, resulting in an amplification of responses. For example, the proinflammatory cytokines IL-1, IL-6 and TNFα, which are involved in the so-called acute phase response, synergize to mediate inflammation, shock and even death in response to infectious agents

Cytokines may resemble hormones at first sight, since both are soluble mediators that serve as a means of intracellular communication. However, there are important differences between the effects of these two families of mediator molecules. Whereas hormones can be easily detected in the circulation, having endocrine (systemic) effects, most cytokines are released locally. Moreover, hormones are released as a result of internal physiological variation and, therefore, are important in maintaining a situation of homeostasis. Cytokines, on the other hand, are produced in short bursts, following external insults, as well as during developmental and effector phases of immune responses, thereby modulating the function of adjacent cells or the cells that produce them. Due to their short half-life in the circulation, cytokines are generally difficult to detect in serum or plasma, although there are a number of exceptions. For example, during acute inflammation or septic shock, IL-1, IL-6 and TNFα orchestrate a series of events that modulate the acute phase response and can readily be detected in human serum or plasma. However, the systemic effects of these cytokines may differ significantly from the effects in the local sphere of influence. IL-1, for example, when released by tissue

macrophages, is a co-stimulatory factor for T-cell activation, whereas high systemic levels of IL-1 result in fever, leukopaenia and, as mentioned above, even shock.

Cytokines can be classified into a number of limited categories (Table 1), broadly based on their functional properties, i.e.:

- cytokines that are involved in innate immunity;
- in the regulation of lymphocyte function;
- in the regulation of haematopoiesis;
- those having anti-inflammatory modes of action;
- those having proinflammatory modes of action;
- cytokines with chemoattractant properties.

As is clear from this list, which is not exhaustive, many cytokines feature in more than one category, which is in line with the notion that redundancy serves the purpose of the immune system to mount an effective and rapid response following inflammation or antigenic challenge. The concomitant release of several cytokines will lead to a rapid mobilization of effector cells at the place of injury or insult to produce the desired biological effects. Cytokines with a broad spectrum of action, such as IL-1 and IL-6, are involved in natural immune responses, which constitute the first line of defence of the host against antigenic challenge, but also have proinflammatory effects and exert stimulatory effect on the activation of T cells, as well as on the differentiation and growth of B cells. Conversely, in order to prevent an exacerbated response, resulting in tissue damage or destruction, many cytokines have down-regulatory effects on their own secretion, and on the production of other cytokines to dampen the immune response. Examples of such cytokines are IL-10 and transforming growth factor β (TGFβ), which have many immunosuppressive effects and which affect a wide range of target cells. Finally, several cytokines are usually involved in, and are required for, the generation of an appropriate immune response, and it seems difficult to bestow a more or less prominent role on each of them in view of their interregulatory effects, although there may be a certain hierarchy in the action of cytokines, especially with respect to kinetics of production. Taken together, the concomitant inducing and inhibitory effects of many cytokines constitutes the basis for the creation of overlapping cytokine networks, which are able tightly to regulate the immune response.

◆◆◆◆◆ ASSAYS FOR MEASURING CYTOKINE PRODUCTION

Several assays have been developed during the past decade to detect and quantify cytokines in sera, body fluids and culture supernatants, including bioassays, immunoassays, such as the enzyme-linked immunosorbent assay (ELISA), intracellular cytokine staining assay and ELISPOT assay, as well as assays to quantitatively detect transcripts in cytokine-producing cells, such as

Table 1. Classification of cytokines based on functional properties

Cytokine	Function	Produced by
Cytokines involved in innate immunity		
IL-1α/β	Mediates host response to infectious agents	Mϕ, DC, fibroblasts, astrocytes
IL-1RA	Natural antagonist of IL-1, blocks IL-1-mediated signalling	Mϕ
TNFα	Mediates host response to infectious agents	Mϕ, T cells
IL-6	Mediates and regulates inflammatory responses	Mϕ, T cells, fibroblasts
Chemokines	Mediate leukocyte chemotaxis and activation	
Cytokines involved in regulation of lymphocyte function		
IL-1	Mediates co-stimulation of T cells	T cells, Mono
IL-2	T-cell growth factor, proliferation of B cells	T cells, NK cells
IL-4	T-cell proliferation, differentiation of Th2 cells B-cell differentiation, IgG4/IgE switching	T cell, mast cells
IL-5	B-cell growth and activation	T cells
IL-6	B-cell proliferation, enhances Ig secretion by B cells	
IL-7	Pre-T and pre-B-cell proliferation, differentiation of LAK and cytotoxic T cells	Bone marrow stromal cells
IL-9	Co-stimulator for mast cell, fetal thymocyte growth	T cells
IL-10	Co-stimulator for T cell, B cell and mast cell growth, down-regulation MHC class II on Mono	Mono, T cells, keratinocytes
IL-12	Differentiation of Th1 cells, induction of IFN-γ by T cells and NK cells	Mono, B cells
IL-13	B-cell differentiation, IgG4/IgE switching	T cells, B cells, mast cells
IL-15	T-cell, mast cell growth factor	Mono
IL-18	Induction of IFN-γ production by T/NK cells	Mono
TNFβ	Stimulation of T-cell growth	T cell, B cells
IFN-γ	Mϕ/NK cell activation, upregulation MHC class I/II on Mono	T cells, NK cells

Cytokines involved in the regulation of haemopoiesis		
IL-3	Synergistic action in haemopoiesis	T cells, thymic epithelial cells
IL-5	Growth/differentiation of eosinophils	T cells, mast cells
IL-6	Growth/differentiation of megakaryocytes	
G-CSF	Growth/differentiation of granulocytic lineage	Mϕ, T cells
GM-CSF	Growth/differentiation of myelomonocytic lineage	Mϕ, T cells
SCF (ckit-L)	Growth/differentiation of haemopoietic precursor cells	Bone marrow stromal cells
Erythropoietin	Growth/differentiation of erythroid progenitor cells	Kidney cells cells
Cytokines having anti-inflammatory effects		
IL-4	Inhibition of proinflammatory cytokines by Mono	
IL-13	Inhibition of proinflammatory cytokines by Mono	
IL-10	Inhibition of proinflammatory cytokines by Mono, inhibition MHC class II expression Mono	
TGFβ	Inhibition of proinflammatory cytokines by Mono, inhibition of T cell growth	Chrondrocytes, Mono, T cells
Cytokines having proinflammatory effects		
IL-1α	Participates in acute phase response	
IL-6	Induction acute phase proteins in the liver	
TNFα	Participates in acute phase response	
IL-16	Migratory response in $CD4^+$ T cells/Mono/EO	$CD8^+$ T cells
Chemokines		
IL-8	Chemotactic for neutrophils, T cells	Mϕ, other
MCP-1	Chemotactic for Mono	Mϕ, other
MIP-1a	Chemotactic for Mono, T cells, Eo	Mϕ, other
MIP-1b	Chemotactic for Mono, T cells	T cells, B cells, Mono
RANTES	Chemotactic for Mono, T cells, Eo	T cells, platelets
Chemotactin	Chemotactic for T cells	T cells
Eotaxin	Chemotactic for eosinophils, basophils and T cells	Epithelial cells, Mϕ
SDF-1	Chemotactic for T cells	Stromal cells

DC, dendritic cells; EO, eosinophils; Mono, monocytes; Mϕ, macrophages

the RNase-protection assay and the quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) assay. An overview of the advantages and disadvantages of the assays described in this chapter is given in Table 2. In the following sections, the ELISA, intracellular cytokine staining assay and quantitative RT-PCR assay are described in detail. The ELISPOT assay and ELISA for measuring mouse cytokines is described in Chapter II.2.4.

Table 2. Comparison of assays for the measurement of cytokines

Bioassay
Very sensitive
Active form of cytokine is detected
Can be performed in the absence of standard: activity expressed in arbitrary biological units
Not specific and requires use of neutralizing antibodies to demonstrate specificity
Large series of dilutions required to fit results on dose-dependent S-shaped curve
Requires labour-intensive tissue culture for relevant responder cells

ELISA
Specific, but non-active form of cytokine is also detected
Results are easy to fit onto dose-dependent S-shaped curve
Different modes of activation, including antigen-specific stimulation, can be used
Less sensitive than bioassay
Sensitivity is dependent on the capture antibody
Results can be affected by sequestration of cytokines from biological samples by (soluble) cytokine receptors or by inducing/inhibitory effects mediated by simultaneously produced cytokines.

Intracellular staining
Specific, but not very sensitive for certain cytokines
Sensitivity dependent on antibody and requires selection of suitable antibodies that detect chemically fixed cytokines
Use in flow cytometry permits analysis of frequency and phenotype of cytokine-producing cells, as well as kinetics of cytokine production
Simultaneous detection of several cytokines produced by same cell
Results are not affected by inducing/inhibitory effects of simultaneously produced cytokines
Although theoretically possible, difficult to use under conditions of antigen-specific stimulation
Requires viable cells at the end of the stimulation

Competitive RT-PCR
Specific and extremely sensitive
Detects cytokine mRNA only and not (secreted) protein
Technically demanding and calibration difficult

Activation Procedure

Since the production of cytokines is tightly regulated, at the mRNA, as well as the protein level, cell populations need to be activated for an appropriate amount of time to allow detection of cytokine transcripts or production of protein. The activation procedures used in the three assays described in this chapter are nearly identical, and will be described first.

Protocol

1. Stimulate 10^6 T cells ml^{-1} with either of the following agents (see notes 1 and 2):
   - PMA (1 ng ml^{-1})/A23187 (500 ng ml^{-1}) (Cal biochem, La Jolla, CA; catalogue Nos 524400 and 100105, respectively);
   - PMA/soluble anti-CD3 monoclonal antibody (mAb) (1 µg ml^{-1}).
   - anti-CD28 mAb (1 µg ml^{-1})/coated anti-CD3 mAb (coat plates with anti-CD3 mAb by incubation with 10 µg ml^{-1} anti-CD3 mAb diluted in PBS for 12–16 h at 4°C and wash twice with 100 µl culture medium prior to use);
   - combination of mitogenic anti-CD2 mAb in the presence or absence of anti-CD28 mAb.

   Use 24-well (final volume 1 ml), 48-well (500 µl) or 96-well (200 µl) plates.
2. Incubate the cells at 37°C, 5% CO_2.
3. *For ELISA* (see note 3): harvest the supernatants after 48 h of incubation, spin at 1200 rpm for 10 min to remove residual cells, aliquot and store at –80°C prior to analysis.
4. *For RT-PCR:* harvest the cells after 4–8 h of incubation, spin at 2000 rpm for 10 min at 4°C, carefully remove supernatant, snap freeze the pellet in liquid nitrogen and store at –80°C prior to further processing or lyse pellet as described on page 642.
5. *For intracellular staining* (see notes 3 and 4): after 4 h of incubation, add 10 µl of Brefeldin A and incubate the cells for an additional 2 h at 37°C, 5% CO_2.
6. Harvest cells and put on ice, prior to analysis.

Notes and recommendations

1. Various modes of stimulation have been reported for *in vitro* induction of cytokines by T cells and monocytes. Polyclonal activators, such as the combination of phorbol ester and calcium ionophore or ionomcyine, respectively, staphylococcus enterotoxin B (SEB), lipopolysaccharide (LPS) have been useful in characterizing cytokine-producing cells. However, the cytokine production profile may depend on the mode of activation and it is therefore recommended that different stimulation protocols be compared, including activation of the cells with anti-CD3 and anti-CD28 mAbs, which resembles antigen-specific stimulation conditions.
2. For optimal stimulation of peripheral blood mononuclear cells (PBMCs) and human T-cell clones it is recommended to use Yssel's medium (Yssel *et al.*, 1984) (Irvine Scientific, Santa Ana, CA; catalogue No. 99223).

3. Since cytokines are secreted with different kinetics, T-cell clones should be stimulated for at least 48 h before harvesting the supernatants for ELISA purposes. Culture supernatants can be frozen prior to cytokine analysis. Similarly, cytokine standards should be aliquoted and kept frozen. However, repetitive freeze/thaw cycles should be avoided, since most cytokines, in particular IFN-γ, will degrade rapidly and will no longer be detectable. For intracellular staining, 4–8 h is optimal (see also page 632).
4. Agents that block intracellular protein transport, such as Brefeldin A and Monensin have dose- and time-dependent cytotoxic effects and it is not recommended to have both agents included in the cultures for >6 h. Since Monensin has been found to induce the intracellular production of IL-1 and TNFα in monocytes within 30 min of activation, the use of Brefeldin A, instead of Monensin, is preferred. A stock solution of 10 mg ml^{-1} Brefeldin A (Epicentre Technologies Madison, WI; catalogue No. B905MG. Sigma, St Louis, MS; catalogue No. B7651) is made in dimethylsulfoxide (DMSO), diluted in phosphate buffered saline (PBS), and Brefeldin A is added at a final concentration of 10 μg ml^{-1} to the cell suspension. Brefeldin is known to be toxic: avoid contact with skin, eyes and mucous membranes.

◆◆◆◆◆◆ ELISA

This section describes the antibody-sandwich ELISA system, which is used for the detection of cytokines present in the culture supernatant of activated T cells. Although generally not as sensitive as a bioassay, the major advantage of the ELISA is its specificity, enabling the detection of soluble proteins in complex mixtures, which often have overlapping activities or counterregulatory activities, and which are therefore extremely difficult to detect, based on their biological activities alone. To detect a cytokine in solution, plates are coated with the first anticytokine mAb that functions as a catcher and to which the cytokine will be bound. After removal of unbound cytokine, the second anticytokine (detection) mAb is added, which is usually biotinylated and recognizes a different epitope on the cytokine, followed by a washing step and the subsequent addition of an enzyme–streptavidin conjugate. After removal of unbound conjugate, substrate is added, the hydrolysis of which by the enzyme conjugate is proportional to the amount of cytokine present in the solution.

Reagents and equipment

- Cytokine-containing supernatants; cytokine-specific capture mAb; biotin-labelled, cytokine-specific tracer mAbs; PBS; dH_2O.
- Culture medium to dilute cytokine-containing samples and cytokine standards:

Coating buffer:	Carbonate–bicarbonate buffer, pH 8.6.
Washing buffer:	PBS, supplemented with Tween-20 (0.05%).
Tween buffer:	PBS, supplemented with BSA (0.1%) and Tween-20 (0.05%).
Blocking buffer:	PBS, supplemented with BSA (2%).

Conjugate:	Streptavidine–alkaline phosphatase (AP) conjugate (Southern Biotechnology Associates, Birmingham, AL; catalogue No. 7100-04).
ELISA substrate:	Sigma 104 phosphatase substrate (Sigma; catalogue number 104-0).
Substrate buffer:	Diethanol amine (97 ml in 800 ml dH_2O) 0.2g NaN_3 0.1g $MgCl_2 \cdot 6H_2O$ Adjust to pH 9.8 with HCl and adjust volume to 1 l with dH_2O.

- H_2O_2; PVC U-bottom 96-well plates; Immunolon I U-bottom 96-well plates; Immunolon II U-bottom 96-well plates (Dynatech; catalogue No. 011-010-3450); microtitre plate reader–spectrophotometer with 405-nm filter, or spectrofluorometer (Dynatech; catalogue No. 011-970-1900) with 365-nm excitation filter and 450-nm emission filter.

All reagents should be at room temperature before use in the ELISA. The optimal working concentrations of all antibodies should be determined for each ELISA. A list of monoclonal and polyclonal antibodies suitable for the detection of cytokines is given in Table 3.

Protocol

The procedure for activating cells is given on page 627.

1. Coat 96-well assay plate with 100 µl catcher antibody diluted in carbonate buffer.
2. Seal plate with lid or parafilm and incubate for 2 h at 37°C or overnight at 4°C. Plates can be stored for several months.
3. Wash wells three times with washing buffer and flick plates dry on absorbent tissue.
4. Add 300 µl of blocking buffer, incubate for 30 min and flick plates dry on absorbent tissue. Do not wash.
5. Dilute the samples and cytokine standard (aliquots stored at –80°C) in Tween buffer, mix well and add 100 µl per well in duplicate at appropriate dilutions. Recommended concentrations are given in Table 3.
   - Standard curve: dilute in separate plate in 1 : 2 dilutions to cover a 1000–20 pg ml^{-1} range and add 100 µl per well in duplicate.
   - Unknown samples: dilute as needed (see Notes) and add 50 µl per well
6. Incubate plates overnight at room temperature.
7. Wash three times with washing buffer and flick plates dry on absorbent tissue.
8. Dilute the biotin-conjugated anticytokine detection mAb in Tween buffer and add 100 µl per well.
9. Incubate for 1–2 h at room temperature.
10. Wash three times with PBS and flick plates dry on absorbent tissue.

11. Prepare streptavidin–alkaline phosphatase conjugate in Tween buffer and add 100 µl per well.
12. Incubate for 1 h at room temperature.
13. Wash three times with washing buffer and flick plates dry on absorbent tissue.
14. Prepare the ELISA substrate (dilute 1 mg ml^{-1} ELISA substrate in 100 ml substrate buffer; prewarm at 37°C and add 100 µl per well.)
15. Incubate for 30 min at 37°C to let colour develop and read the OD at 405–430 nm using a spectrophotometer or spectrofluorometer.
16. Use the Softmax program (Molecular Devices Corporation, Menlo Park, CA) or comparable program to analyse the data.

Table 3. Cytokine-specific mAbs for ELISA assay of human cytokines

	High stand conc.		Coating mAb		Detecting mAb	
Cytokine	(ng ml^{-1})	Plate type*	Designation	Conc. (µg ml^{-1})	Designation	Source† coat/det.
IL-2	5	2	MQ1-17H12	5	BG-5	1/3
IL-3	1	1	BVD8-3G11	5	BVD3-IF9	1/1
IL-4	2	1	8D4-8	5	MP4-25D2	4/1
IL-5	2	1	JES-39D10	5	JES1-5A10	1/1
IL-6	5	2	MQ20-39C3	5	MQ2-13A5	1/1
IL-7	2	2	BVD10-40F6	10	BVD10-11C10	1/1
IL-10	5	2	JES8-9D7	10	JES3-12G8	1/1
IL-13	2	2	JES10-35G12	50	JES10-2E10	1/1
G-CSF	5	2	BVDE13-3A5	50	BVD11-37G10	2/2
GM-CSF	5	1	BVD2-23B6	5	BVD11-21C11	2/2
TNFα	5	2	MP9-20A4	25	GMO1-1782	1/1
IFN-γ	5	2	A35	25	B27	3/3

* 1, Immulon 1 microtitre plates; 2, Immulon 2 microtitre plates (Dynatech).

† This list is by no means exhaustive, but shows a panel of mAbs and Abs generated and used at DNAX, Palo Alto, CA. 1, DNAX, ATCC Patent Deposit (Budapest Treaty; permission must be obtained from DNAX for acquisition of cell lines from this source); 2, DNAX, ATCC General Deposit (no permission is required from DNAX for acquisition of cell lines from this source); 3, Diaclone, Besançon, France; 4, Pharmingen, San Diego, CA. In view of the overwhelming commercial supply of purified mAbs for ELISA purposes, readers are referred to the relevant manufacturer.

Notes and recommendations

It is recommended that several dilutions of cytokine-containing supernatans are made in order to be able to measure at the linear part of the standard curve.

It is important to note that the presence or absence of detectable levels of cytokines in clinical samples, such as serum and plasma, does not always correlate with their functional activity. Cytokines are often bound to non-specific serum proteins or to soluble cytokine receptors, which will sequester free cytokine from the peripheral circulation and might result in

a decrease in detectable cytokine levels, as measured with ELISA. However, the biological significance of free versus complexed cytokines is not yet clear. In addition, circulating anticytokine antibodies, notably in the serum of those who have received cytokine or anticytokine therapy, may decrease levels of free cytokine, although they may still be detectable by cytokine-specific mAbs. Conversely, cytokine receptor antagonists may specifically compete with the cytokine for binding to its receptor. For example, despite high levels of IL-1 in certain serum samples, possible receptor occupancy by IL-1RA will prevent IL-1-mediated responses and, therefore, will prevent a meaningful analysis of its functional activity (Arend, 1993).

The half-life of many cytokines in serum is very short (generally of the order of minutes), which is one of the reasons why cytokine levels in the peripheral circulation are usually very low in healthy individuals, as measured by ELISA. Certain cytokines (notably IL-1, IL-6, IL-10 and TNFα) may be detectable in the serum as well, following sepsis, trauma or an acute or chronic inflammatory state. However, it should be kept in mind that, due to existing cytokine networks, even in above-mentioned pathological situations, the presence in excess of certain cytokines may result in the suppressed production of another cytokine and a failure to detect the latter.

Finally, due to the possible presence of proteases or other (unknown) factors, present in clinical samples, as well as culture supernatants, cytokines may be unstable and subject to degradation. Therefore, it is recommended that samples are aliquoted and stored at –80°C prior to analysis, especially when more or less prolonged periods of storage are required.

◆◆◆◆◆◆ INTRACELLULAR CYTOPLASMIC STAINING

The method of staining with cytokine-specific mAbs for the analysis of intracellular cytokines in suspension by immunofluorescence using an ultraviolet (UV) microscope was originally described by Andersson *et al.* (1990). Subsequently, it was shown that the presence of intracellular cytokines could be detected using a FACS flow cytometer (Jung *et al.*, 1993; Assenmacher *et al.*, 1994), and recent improvements in the fixation procedure (Openshaw *et al.*, 1995) and the use of Brefeldin as a protein transport inhibitor (Picker *et al.*, 1995) have made the method even more suitable for multiparameter flow cytometry analysis. The advantage of this method over the measurement of cytokines in culture supernatants of activated cells is that it enables the determination of the frequency of cytokine-producing cells, as well as the kinetics of cytokine production. Moreover, in combination with cell surface staining, the method can be used to identify the phenotype of cytokine-producing cells in a population of non-separated cells, whereas the development of fluorochromes with different emission wavelength and flow cytometers, equipped with dual lasers, has permitted the simultaneous detection of several cytokines, provided the mAbs are conjugated directly with the appropriate fluorescent dyes.

Critical parameters for successful intracellular cytokine staining include the choice of a proper fixation and permeabilization protocol, the inclusion of a protein transport inhibitor, cell type and activation protocol and kinetics of activation. Each of these parameters is discussed below (Fig. 1).

The principle and sensitivity of the method depends on the availability of mAbs that recognize natural cytokines, in spite of the fixation and permeabilization procedure. Fixation of the cells is required for subsequent treatment with detergent, and ideal fixatives preserve the morphology of the cells and antigenicity of the cytokines with minimal cell loss. Optimal results have been obtained with formaldehyde (Openshaw *et al.*, 1995) and, although this fixative is less efficient at cross-linking and changing the tertiary structure of protein than is glutaraldehyde, its effects are more gentle and fixation with formaldehyde generally preserves a high degree of antigenicity. Subsequent permeabilization of the cell membranes with the detergent saponin allows fluorochrome-labelled cytokine-specific mAbs to penetrate through the cell membrane, cytosol and the membranes of the Golgi apparatus and endoplasmic reticulum.

Since cytokines are produced with different kinetics, optimal time points for the analysis of activated cytokine-producing cells may vary, depending not only on the type of cytokine to be analysed, but also on the mode of stimulation. The peak production of most cytokines, including IL-2, IL-4 and TNFα, by T-cell clones, following stimulation with TPA and A23187, is within the first 6–7 h, whereas others, such as IFN-γ and IL-10 have more long-lasting kinetics. Interleukin-13 is produced early (2 h) following activation, but this cytokine also continues to be produced up to 72 h. Analysis of the cells 4–6 h after activation results in a characteristic staining of the Golgi apparatus and endoplasmic reticulum, which is bright for cytokines such as IL-2, TNFα and IFN-γ. Importantly, the addition of agents that inhibit cellular transport systems, such as Brefeldin A or Monensin, results in accumulation of newly synthesized protein in the Golgi complex. Although the cellular integrity changes, following treatment with intracellular protein transport inhibitors, resulting in a disappearance of Golgi staining and a homogeneously fluorescing cells, their use generally gives a brighter staining pattern and enables the detection of intracellular cytokines that stain only weakly with anticytokine mAbs, such as IL-4 and IL-5. However, as these compounds have dose- and time-dependent cytotoxic effects, their use should be limited. Brefeldin A is less toxic than Monensin and should be added to the cultures between 2 and 6 h before harvesting of the cells. Extensive analysis of peripheral blood mononuclear T cells and cloned T-cell lines, in order to determine optimal kinetics, has shown that activation for 6 h, including 2 h of Brefeldin A treatment, resulted in a higher frequency of IL-2, IL-4 and IFN-γ-producing cells than did activation for 24 h including 2, 6 or 10 h Brefeldin A treatment (H. Yssel, unpublished results). It should be kept in mind, however, that the method provides only a 'snapshot' of cytokine production, and that the observed frequency of cytokine-producing cells is affected by multiple variables, such as activation conditions and the state of cell cycling.

Intracellular staining for detection of cytokines in tissue sections has been described in detail by Litton *et al.* (1997).

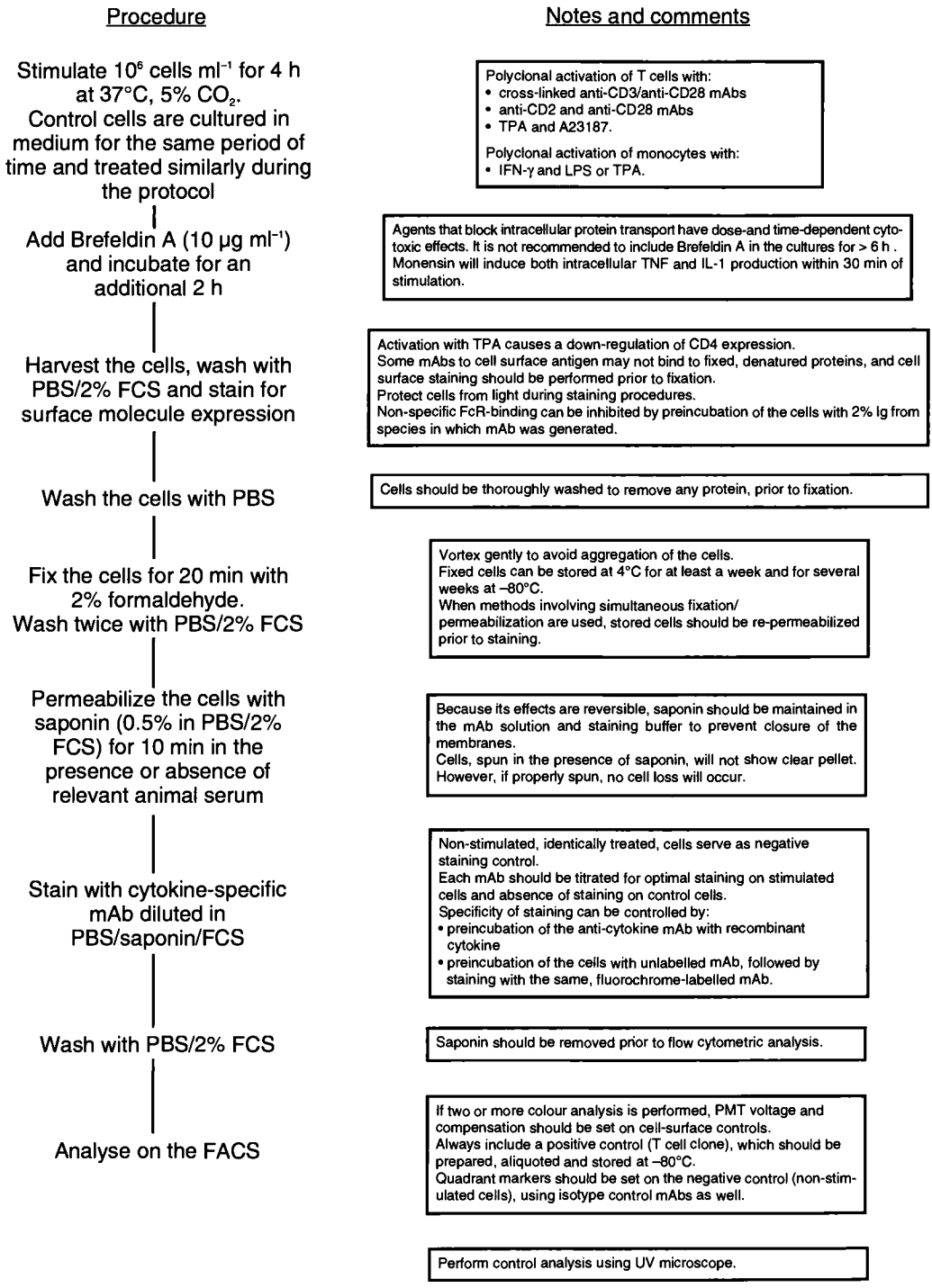

Figure 1. Flowchart for intracellular cytokine staining.

Table 4. Human cytokine-specific mAbs for intracellular staining

Cytokine	mAb	Isotype*	Source†
IL-1α	1277-89-7	mIgG1	1
	1277-89-29	mIgG1	1
	B-A15	mIgG1	2
IL-1β	2D8	mIgG1	1
	1437.96-15	mIgG1	1
IL-2	MQ1-17H12	rIgG2a	3/6
	BG-5	mIgG1	2
IL-3	BVD8-3G11	rIgG2a	3/6
	BVD3-IF9	rIgG2a	3/6
IL-4	8D4-8	mIgG1	3
	MP4-25D2	rIgG1	3/7
	B-R14	mIgG1	2
	3010.2	mIgG1	4
IL-5	JES-39D10	rIgG2a	3/6
IL-6	MQ2-6A3	rIgG2a	3/6
IL-8	NAP-1	mIgG1	5
IL-10	JES8-12G8	rIgG2a	3/6
	JES3-19F1	rIgG2a	3/6
	B-T10	mIgG1	2
IL-12	B-5E9	mIgG1	2
IL-13	JES8-30F11	rIgG2a	7
	JES8-5A2	mIgG1	3/4/6
	BB-13	mIgG1	2
GM-CSF	BVD2-21C11	rIgG2a	3/6
G-CSF	BVD13-3A5	rIgG1	3/6
	BVD11-37G1	mIgG1	3/6
TNFα	mAb 1	mIgG1	3
	B-D9	mIgG1	2
	MP9-20A4	rIgG1	6
IFN-γ	B27	mIgG1	2
	4S.B3	mIgG1	3
	25723.11	mIgG1	4

* m, Mouse mAb; r, rat mAb.

† Commercial sources are: 1, Immunocontact, Zürich, Switzerland; 2, Diaclone, Besançon, France; 3, PharMingen, San Diego, CA; 4, Becton Dickinson, San Jose, CA; 5, Novartis, Vienna, Austria; 6, DNAX, Palo Alto, CA, ATCC Patent Deposit (Budapest Treaty) (permission must be obtained from DNAX for acquisition of cell lines from this source); 7, DNAX, Palo Alto, CA, ATCC General Deposit, (no permission is required from DNAX for acquisition of cell lines from this source).

Reagents and equipment

- *Washing buffer.* PBS without Ca^{2+} and Mg^{2+}, supplemented with 2% heat-inactivated fetal calf serum (FCS) and 0.1% (w/v) sodium azide (NaN_3) (see note 1). Adjust buffer to pH 7.4 and store at 4°C.
- *Permeabilization buffer.* Washing buffer, supplemented with 0.5% saponin (Sigma; S7900). Make stock solution of 10% saponin in PBS (pH 7.4), dissolve at 37°C, filter solution through a 0.2-µm filter and store at 4°C (see note 1).
- *Formalin* (37% formaldehyde solution; Sigma F1635). Dissolve 10.8 ml formalin in 89.2 ml PBS, filter to remove particles and store at 4°C (see note 1).
- *Cytokine-specific mAbs* (preferentially directly conjugated with FITC, phycoerythrin (PE) or Cy5). A list of mAbs suitable for intracellular staining is given in Table 4.
- *Second step FITC-labelled antibodies.* Horse-anti-mouse IgG-FITC/PE (Vector, Burlinghame, CA; FL 2000/EL 2000). Rabbit-anti-rat IgG-FITC (Vector, FL 4000).
- *Analysis by UV microscope.* Bio-Rad adhesion slides (Bio-Rad Laboratories, München, Germany; catalogue No. 180-7001); plastic box with humid paper tissue; dH_2O; slideholder to wash slides; Vector stain enhancer; cover slides; nail polish.
- *Analysis by FACScan flow cytometer.* Vortex; centrifuge with rotor to spin microtitre plates; 96-well V-bottom plates (Falcon, Becton Dickinson); FACS tubes (Becton Dickinson).

Protocol: Staining for flow cytometry

The procedure for activating cells is given on page 627.

If cell-surface staining is performed, start with step 1; for intracellular cytokine staining without cell-surface staining, start with step 7 (see note 2).

1. Transfer the stimulated cells to a 15-ml centrifuge tube and wash twice with 1 ml ice-cold washing buffer. Washing with larger volumes will result in cell loss. Spin the cells at 1000 rpm for 5 min.
2. To block non-specific binding to FcR, add 20 µl of 2% serum (from same animal species as the mAbs used for the immunofluorescence staining) to the cell pellet, and incubate cells for 15 min.
3. Wash cells twice with 1 ml cold washing buffer.
4. Add 20 µl directly conjugated anti-cell surface mAbs to the cell pellet.
5. Incubate for 15 min on ice.
6. Wash cells twice with 1 ml ice-cold PBS and proceed to step 8.
7. Transfer the stimulated cells to a 15-ml centrifuge tube and wash twice with ice-cold PBS.
8. Resuspend the cells at 2×10^6 cells ml^{-1} in cold PBS and add an equal volume of 4% formaldehyde. Mix well to prevent aggregates.
9. Incubate for at least 20 min at room temperature.
10. Spin the cells at 1000 rpm for 5 min and wash once more with 1 ml cold PBS. At this point the cells can be resuspended in PBS and

stored at 4°C in the dark for 1 week or aliquoted and stored at –80°C for at least 1 month prior to analysis (see note 3).

11. Resuspend the cells in washing buffer and transfer at ± 10^5 cells per well to a 96-well V-bottom microtitre plate and spin at 1000 rpm for 2 min.
12. Remove supernatant by flicking the plate, blot the plate dry on a paper tissue and resuspend the cells by vortexing gently.
13. Add 150 µl permeabilization buffer to the cells and incubate for 10 min at room temperature. A final concentration of 2% serum (as described under step 2) can be added to block non-specific FcR binding of mAbs.
14. Spin the cells at 1000 rpm for 2 min and remove supernatant.
15. Add 20 µl cytokine-specific mAb solution to the cell pellet (see note 4).
16. Incubate cells for 20 min at room temperature.
17. When using non-conjugated mAbs, proceed to step 18. For FITC, PE or Cy5 conjugated mAbs, proceed to step 22.
18. Add 150 µl permeabilization buffer, centrifuge the plate at 1000 rpm for 2 min, remove the supernatant and resuspend the cells.
19. Repeat washing step.
20. Add 20 µl per well of an appropriate dilution of the second conjugated mAb.
21. Incubate cells for 30 min at room temperature.
22. Add 150 µl permeabilization buffer, centrifuge the plate at 1000 rpm for 2 min, remove supernatant and resuspend the cells.
23. Repeat washing step.
24. Wash once with washing buffer, resuspend in 200–300 µl PBS and analyse on the FACS flow cytometer as soon as possible.
25. For instrument control and compensation settings, refer to the manufacturer's instructions (see reference Lanier and Recktenwald (1991) and notes 3, 5 and 6).

Protocol: Staining for microscopic analysis using Bio-Rad slides

1. Wash the protective green layer off the Bio-Rad adhesion slide with dH_2O and incubate the slide for 5 min in PBS.
2. Add 2×10^4 to 10^5 cells in a volume of 15 µl to each slot and incubate for 10 min at room temperature. Check cell density using inversion microscope.
3. Gently wash the slide with washing buffer to remove non-adherent cells.
4. Add 20 µl fixation buffer to each slot and incubate for 15 min.
5. Gently wash the slide with washing buffer.
6. The staining procedure used for Bio-Rad slides is identical to that for flow cytometric analysis (see note 6).

Notes and recommendations

1. NaN_3 is known to be toxic, saponin is an irritant and formaldehyde is a suspected carcinogen. Avoid contact with skin, eyes and mucous membranes. Dispose of formaldehyde in a container for treatment; do not put formaldehyde-containing waste down the sink.
2. The staining procedure for flow cytometric analysis is performed in 96-well V-bottom microtitre plates. However, it is recommended that cell-surface molecules are stained in 15-ml centrifuge tubes, followed by fixation and transfer of the cells to microtitre plates. All steps during cell-surface staining are carried out at 4°C, in the absence of saponin. Since some mAbs may not bind to fixed, denatured, protein, cell-surface staining should be carried out before fixation of the cells. Staining for intracellular cytokines is performed at room temperature in the presence of saponin. All incubation periods during the staining procedures are carried out in the dark.
   - Make the antibody dilutions in PBS/2% FCS/NaN_3/0.5% saponin. For each mAb, the optimal titre has to be determined. Generally, final concentrations of mAbs are 1–5 μg per 10^6 cells. PE-conjugated mAbs are not recommended for analysis using a UV microscope, because of the quick fading of the dye. Use Rhodamin-conjugated antibodies instead.
   - Centrifugation of the antibodies at high speed in a microcentrifuge to remove aggregates will improve the quality of the staining.
   - If non-conjugated anticytokine mAbs, from different species are used, two-colour analysis can be performed. Although cytokine-specific staining signals generally tend to be higher, increased fluorescence backgrounds are often observed, which requires rigid blocking of non-specific mAb binding.
3. *Positive control.* Samples of a resting and activated T clone with a Th0 cytokine production profile can be fixed, aliquoted in PBS, stored at –80°C for several weeks and used in the staining procedure as negative control (to adjust flow cytometer instrument settings) and positive control, respectively.
4. *Negative control.* Non-stimulated cells, which have been treated identically during the staining procedure, serve as a negative control. Prior to their use in the staining procedure, each anticytokine mAb should be titrated at a concentration that gives optimal staining of activated cells and no staining of non-stimulated control cells. Furthermore, it is recommended that one of the following controls is used to confirm the specificity of staining:
   - *Ligand-blocking control.* Pre-incubate mAb with excess of recombinant cytokine in permeabilization buffer at 4°C for 30 min. It is recommended to use at least a 50-fold molar excess of cytokine over mAb. Proceed to step 15 of the staining protocol.
   - *Non-conjugated mAb control.* Pre-incubate cells after step 14 of the staining protocol with non-conjugated mAb, diluted in permeabilization buffer, at 4°C for 30 min. Wash cells twice and proceed to step 15 of staining protocol.
   - *Isotype control.* Use an irrelevant mAb of the same isotype as the anticytokine mAbs in the staining procedure. Isotype controls can be used to adjust instrument settings, including quadrant markers and compensation of the flow cytometer. In cases where isotype controls give brighter

staining than the anticytokine mAb, rely on non-activated cells as the negative control.

5. It is important to note that when the intracellular staining method, using flow cytometry, is introduced in the laboratory, its results should be confirmed by UV microscope analysis. Intracellular cytokine staining of cells that have been activated for less than 6 h in the absence of Brefeldin A will result in distinct staining of the Golgi apparatus and endoplasmic reticulum, whereas after longer activation periods cell surface cytokine staining may also be detected. Microscopic analysis of cells activated in the presence of Brefeldin A show homogenous staining throughout the cytoplasm, as a result of disintegration of Golgi apparatus and endoplasmic reticulum (Openshaw *et al.*, 1995).
6. All incubations using Biorad slides are carried out at room temperature. When using Bio-Rad adhesion slides, keep the slides in a wet chamber (filter paper soaked with dH_2O) during incubations in order to prevent drying out of the slots. After each washing step, remove medium remaining between the slots with a piece of absorbent paper to prevent spillover between adjacent slots. Add 15 µl antibody solution per slot. After staining procedure, add 10 µl Vector stain enhancer, cover the slots with a cover slide, seal off with nail polish and analyse the cells using a UV microscope. (Slides can be stored for months in the dark, if properly sealed.) Slides that contain cells fixed with formaldehyde can be stored at –80°C for several weeks.

◆◆◆◆◆ COMPETITIVE RT-PCR

The reverse transcription polymerase chain reaction (RT-PCR) is a powerful technique for analysing gene regulation at the cellular level (Ferre, 1992). However, the advantage of sensitivity is also a limitation due to the two enzymatic reaction steps. The reverse transcriptase first synthesizes a complementary strand from the RNA with specific, random or oligo dT primers, and this newly synthesized cDNA is subsequently amplified by the Taq polymerase with two specific primers for a sufficient number of times to be analysed. Moreover, due to the exponential nature of the PCR step, the effects of minor variations in reaction conditions can lead to large differences in the results obtained from different samples (Hengen, 1995). Finally, the quantification of a specific cDNA present in the sample is only possible in the exponential phase of the enzymatic reaction, where the amount of amplified targets is a linear function of the starting template (Gilliland *et al.*, 1990).

In order to monitor all these potential variations, many controls have been proposed, from the amplification of an internal housekeeping gene along with the unknown target (Chelly *et al.*, 1988), to serial dilutions of samples that lie outside the standard curve of a known template (Murphy *et al.*, 1993), but the most precise and accurate method remains the competitive PCR (Zimmerman and Mannhalter, 1996). In the latter technique, an external template is mixed at different dilutions with a constant amount of cDNA previously equilibrated with the relevant amount of a constitutively expressed gene. This competitor has the same primer

recognition sites as the cDNA of interest, but has a different amplification size; thus the two amplified products can be easily separated after electrophoresis (Becker-André and Hahlbrock, 1989; Wang *et al.*, 1989). To be really accurate, the competitor must be amplified with the same efficiency as the cDNA of interest (Cottrez *et al.*, 1994). This setting has to be carefully carried out before the adoption of the routine procedure of this method. After the PCR step, the samples are analysed on an electrophoresis gel, the amount of each amplified PCR product is measured, and the ratios plotted on a graph against the molar concentrations of the competitor. The amount of the mRNA present in the sample corresponds to the concentration of the competitor when the ratio of the two amplified molecules is 1.

A known amount of competitor can also be introduced at the reverse transcription step in a single reaction tube along with the other samples in order to control the other enzymatic reaction step (Wang *et al.*, 1989).

Methods

Designing specific primers

The generation of specific primers is a crucial factor in the success of achieving a very accurate competitive PCR (Rappolee, 1990; He *et al.*, 1994). Ideally, the primers for all cytokines should be designed to amplify PCR products that have about the same size. Indeed, since the competitor is amplified by the different primer pairs to give products of the same size, in order to minimize differences in PCR efficiencies between the competitor and the wild-type template the size difference of the PCR products, amplified from the wild-type template and the competitor should not exceed 150 bp (Zimmermann and Mannhalter, 1996).

The primers need to have a high annealing temperature (at least 60°C), and should not give rise to non-specific products that would compete for the specific templates during the PCR. In the same manner, they should distinguish the DNA from the RNA; this could be achieved by designing at least one or both primers across a splicing site.

Validation of primers

The first step is to monitor the expected size of the amplified product. Then, ideally, a specific hybridization with an internal sequence should be performed.

Construction of an internal competitor for quantitative PCR

Rationale

There are many ways to construct PCR competitors (Reiner *et al.*, 1993; Forster, 1994), but the most convenient one when the number of templates is relatively high, as is often the case for cytokines, is to create a

multicompetitor standard by linking together all 5'-primer sequences spaced by a non-specific sequence from all linked 3'-primers (Wang *et al.*, 1989; Siebert and Larrick, 1992), as shown in Fig. 2A. Although there are different ways to link nucleotide sequences, it is recommended to use the overlapping primers–PCR strategy, as shown in Fig. 2B.

The first step is to draw the map of the final construct. Ideally, the number of cytokines should not exceed five or six per competitor (Fig. 2A), it is also better to design some competitors for a specific purpose (e.g. proinflammatory cytokines or Th1/Th2 cytokines). The second step is to design the primers for the construct itself. These primers have 15 nucleotides at the 3'-end, which hybridize specifically on the DNA to be extended, and 25 nucleotides that consist of the primer for the cytokine of interest, which

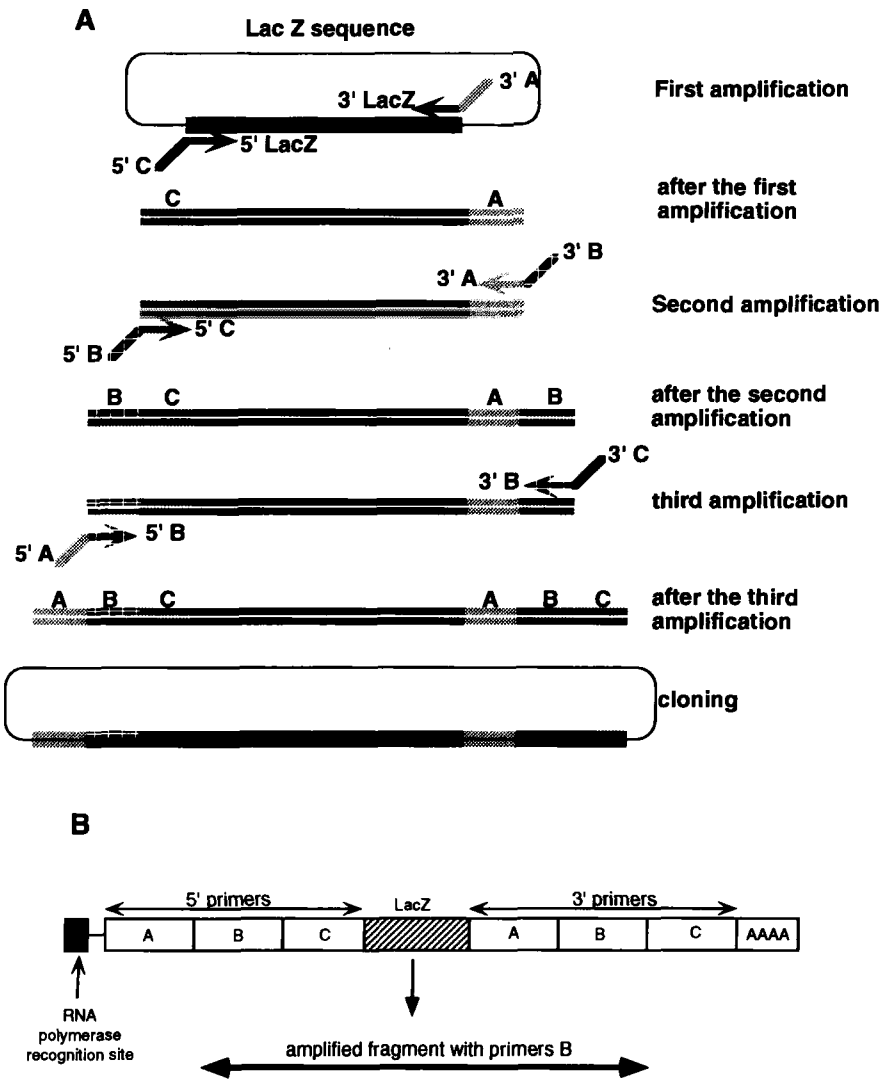

Figure 2. Construction of an internal competitor for quantitative RT-PCR.

needs to be incorporated in the construct. In the final step, it is possible to add a polyA sequence to mix the competitor with the mRNA. Another possibility is to add restriction sites, included in the last primers, in order to achieve directional cloning. Finally, the TA cloning vector system (commercialized by Invitrogen) is very convenient to clone PCR products directly. The competitor is then excised from the plasmid as a double-strand DNA and can be used at the PCR level, or it could also be synthesized as a cRNA from the RNA polymerase recognition site in the plasmid and be used in the RNA or cDNA steps.

Protocol

Plasmid (1 ng) containing the Lac-Z sequence, is added to a total reaction volume of 25 µl of the PCR mixture. After an initial denaturation step at 94°C for 5 min, do two PCR cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 72°C, followed by 35 cycles of 30 s at 94°C, 30 s at 60°C and 30 s at 72°C, followed by a final elongation step at 72°C for 7 min. The amplified product is loaded onto 2% agarose gel in TAE buffer and stained with ethidium bromide. The specific product is recovered by freezing the agarose band at –20°C for 30 min in a 0.5-ml tube, which is then introduced into another clean 1.5-ml Eppendorf tube (a small hole has been previously made on the bottom of the 0.5-ml tube, containing the agarose band with a small needle) and then centrifuged at 13000 rpm for 5 min. Of the 500 µl adjusted volume of the recovered DNA, 1 µl is used in the second step of amplification. This process is repeated enough times to incorporate all primer pairs in the construct. The final fragment is cloned using the plasmid of choice. After bacterial transformation and amplification, the specific band is cut out of the plasmid followed by a purification step on agarose gel using a commercial kit for clean recovery (Wizard PCR prep, Promega). The excised band is then quantified by optical density reading and diluted to be used as an internal competitor.

It must be emphasized that all these amplifications and purification steps must be carried out preferentially in a room different from the one that will be used for RT-PCR analysis, in order to avoid plasmid contamination and PCR product carryover.

Analysis of PCR efficiency for both templates

As mentioned above, the wild-type template and the competitor have to be amplified by PCR with the same efficiency. Several methods for monitoring the efficiency using labelled primers have been described (Celi *et al.*, 1993; Cottrez *et al.*, 1994). Briefly, the wild-type and competitor DNAs are amplified with labelled primers, and the corresponding PCR products are purified, quantified, mixed at different ratios and diluted. The different mixtures are reamplified and the ratios obtained after amplification are measured and compared with the initial ones. An equivalent efficiency should give rise to the same ratios before and after amplification, even after the plateau phase of the PCR.

RNA Preparation

There are two preferred ways to purify the RNA, depending mostly on the amount of material available.

More than 10^6 cells

Total RNA is isolated and purified using RNAzol (Cinna/Biotec, Friendswood, TX) according to the manufacturer's instructions and quantified by means of optical density readings. Pelleted cells are lysed with 1 ml RNAzol, and 0.2 ml chloroform is added. After centrifugation, the aqueous phase is recovered and precipitated with isopropanol (v/v). The pellet is resuspended in 10 µl RNase-free water. It is sometimes necessary to add glycogen or t-RNA as a carrier for better RNA recovery.

Less than 10^6 cells

Total RNA is prepared using an affinity column technique (Glassmax from Gibco BRL, Qiagen, Invitrogen). The Glassmax technique from Gibco BRL is used according to the manufacturer's instructions. Briefly, the cells are pelleted and homogenized in 400 µl of the lysis buffer, and stored at –20°C until use. The homogenate is thawed on ice and pelleted for 5 min at 13 000 rpm with 280 µl cold ethanol. The pellet is resuspended in 450 µl binding buffer and 40 µl ammonium acetate, applied to a column, spun for 20 s at 13 000 rpm, and washed successively with the wash buffer followed by 80% ethanol in water in the same manner. The RNA is then eluted with 30–50 µl RNase-free water, previously heated to 70°C.

Reverse Transcription

RNA (a total of 1 µg; a lower concentration can be used, but for ease of comparison between samples use of a constant number of cells is recommended) is resuspended in 20 µl H^2O and 1 µl of 1 mg/ml^{-1} oligo-dT is added together with 0.1 µl of 40 U ml^{-1} RNAsin (Promega, Madison, WI). The samples are heated at 70°C for 10 min and then cooled to room temperature. The samples are briefly spun down in a microcentrifuge, after which 15 µl of the enzyme mixture (7 µl buffer (5X Superscript buffer; Gibco, BRL, Grand Island, NY), 5 µl of 10 mM dNTP, 1.5 µl of 1 M DTT (Gibco, BRL), 0.1 µl of 40 U ml^{-1} RNAsin (Promega, Madison, WI), and 1.5 µl Superscript II (Gibco, BRL)) is added. The samples are then incubated at 42°C for 1 h, heat denatured at 95°C for 3 min and, after rapid cooling on ice, the volume is adjusted to 200 µl with H_2O. The samples are then stored frozen at –20°C to –80C° until use for RT-PCR amplification.

PCR Amplification

For PCR amplification, 18 µl PCR mixture is placed in each PCR reaction tube (total reaction volume is 25 µl: 1× PCR buffer, 200 µM of each dNTP, 0.5 µM of each primer, 2 mM $MgCl_2$, and 2.5 U per 100 µl Taq polymerase). Then, 2 µl cDNA (0.2–1 µl of the reverse transcriptase reaction, which corresponds to about 2000 cells) is added to each PCR tube. A 5-µl aliquot of each competitor dilution is added to the corresponding reaction tubes (preferably via reversed caps, which are then carefully returned and closed) and the tubes spun for 20 s at 13 000 rpm to mix all the reagents. All PCR samples should be maintained at 4°C. It is essential to prepare the competitor and the first step of the PCR in separate rooms in order to avoid primer–dimer formation, which compete for the specific PCR products. It is recommended that the Anti-Taq antibody (Clontech) is used, or another strategy be employed to ensure a good hot start PCR.

PCR conditions. An initial denaturation step at 94°C for 5 min (necessary for Anti Taq antibody use), is followed by 35 cycles of PCR (30 s at 94°C, 30 s at 60°C, 30 s at 72°C) and then by a final elongation step at 72°C for 7 min.

Adjustments of cDNA Concentrations

The cDNA is adjusted with reference to a housekeeping gene by performing competitive PCR for β-actin or HPRT between the cDNA of interest and the internal competitor DNA (HPRT is expressed at approximately the same levels as IL-2 or IFN-γ, and thus is best used for cytokine gene expression studies). First, each cDNA is amplified in the presence of 10-fold serial dilutions of the internal competitor, in order to evaluate the amount required to achieve equal band intensities for both PCR fragments. This step is followed by two-fold serial dilutions of the internal competitor to determine precisely the amount of cDNA that gives equivalence of the fragments. The volumes of cDNA are adjusted to obtain equal HPRT (or β-actin) gene amplification in each sample, and used to quantify genes of interest by using serial dilutions of competitors in each reaction, in the presence of the specific primers for cytokines (Table 5).

Analysis of PCR Products

The first step of the competitive PCR, which consists of 10-fold dilutions of the competitor, is analysed on a regular agarose gel in order to be able to evaluate visually the corresponding half-dilution working zone. For detailed evaluation the PCR products can be analysed in two different ways.

The easiest and quickest way is to analyse the PCR products on an agarose gel, stained with SYBR-green (Molecular Probes, Eugene, OR), a

Table 5. List of primers for RT-PCR

GENE	Direction	Size	cDNA location	Sequence	Tm
Mouse RT-PCR Primers					
IL-2	5′	255	213	CAG GAG CTC CTG AGC AGG ATG GAG AAT TAC	60
	3′		468	GCA CTC AAA TGT GTT GTC AGA GCC CTT TAG	
IL-4	5′	241	200	ACG AGG TCA CAG GAG AAG GGA CGC CAT GCA	60
	3′		441	TCA TTC ATG GAG CAG CTT ATC GAT GAA TCC	
IL-5	5′	211	160	CCG AGC TCT GTT GAC AAG CAA TGA GAC GAT	60
	3′		371	CCG TCT CTC CTC GCC ACA CTT CTC TTT TTG	
IL-10	5′	258	280	TCC TTA ATG CAG GAC TTT AAG GGT TAC TTG	60
	3′		538	GAC ACC TTG GTC TTG GAG CTT ATT AAA ATC	
IFN-γ	5′	336	230	TGG AGG AAC TGG CAA AAG GAT GGT	60
	3′		566	TTG GGA CAA TCT CTT CCC CAC	
TNFα	5′	384	166	GCG ACG TGG AAC TGG CAG AAG	60
	3′		550	GGT ACA ACC CAT CGG CTG GCA	
HPRT	5′	177	403	GTA ATG ATC AGT CAA CGG GGG AC	60
	3′		580	CCA GCA AGC TTG CAA CCT TAA CCA	
Rat RT-PCR primers					
IL-2	5′	376	48	CAT GTA CAG CAT GCA GCT CGC ATC C	60
	3′		424	CCA CCA CAG TTG CTG GCT CAT CAT C	
IL-4	5′	378	29	TGA TGG GTC TCA GCC CCC ACC TTG	60
	3′		407	CTT TCA GTG TTG TGA GCG TGG ACT C	
IL-10	5′	382	8	GGC TCA GCA CTG CTA TGT TGC CTG C	60
	3′		390	GAA ATC GAT GAC AGC GTC GCA GCT G	
ACTIN	5′	334	231	GCT GCT CAC CGA GGC CCC CCT GAA C	60
	3′		565	CTC TTT GAT GTC ACG CAC GAT TTC	
Human RT-PCR primers					
IL-2	5′	200	261	AAG AAT CCC AAA CTC ACC AGG ATG C	60
	3′		461	CCC TTT AGT TCC AGA ACT ATT ACG TT	
IL-4	5′	300	199	ACT CTG TGC ACC GAG TTG ACC GTA A	60
	3′		499	TCT CAT GAT CGT CTT TAG CCT TTC C	
IL-5	5′	250	251	GGG AAT AGG CAC ACT GGA GAG TCA A	60
	3′		501	TAA AAT GTC CTT CTC CTC CAA AAT C	
IL-10	5′	352	314	ATG CCC CAA GCT GAG AAC CAA GAC CCA	60
	3′		666	TCT CAA GGG GCT GGG TCA GCT ATC CCA	
IFN-γ	5′	494	109	ATG AAA TAT ACA AGT TAT ATC TTG GCT TT	60
	3′		603	GAT GCT CTT CGA CCT TGA AAC AGC AT	
ACTIN	5′	310	231	GCT GCT GAC CGA GGC CCC CCT GAA C	60
	3′		541	CTC CTT AAT GTC ACG CAC GAT TTC	

DNA intercalate dye that is excited at 497 nm and can be analysed using either a scanner Storm (Molecular Dynamics, Sunnyvale, CA) or a 300-nm UV transilluminator linked to image quantification software. Sybr-green is 10-fold more sensitive than ethidium bromide, and the volume of each PCR loading used has to be determined carefully for each cytokine. It is also recommended that the gel is stained after electrophoresis (as specified in the manufacturer's instructions). The scan or the image is then analysed using software such as Image Quant (Molecular Dynamics), which gives an accurate evaluation of the band intensities by creating a graph and analysing the heights and areas of the peaks. The ratios are then plotted against the concentrations of the competitor, and the concentration of each unknown sample is determined for a ratio equal to 1 (Fig. 3). If the molarity of the competitor has not been determined, the results

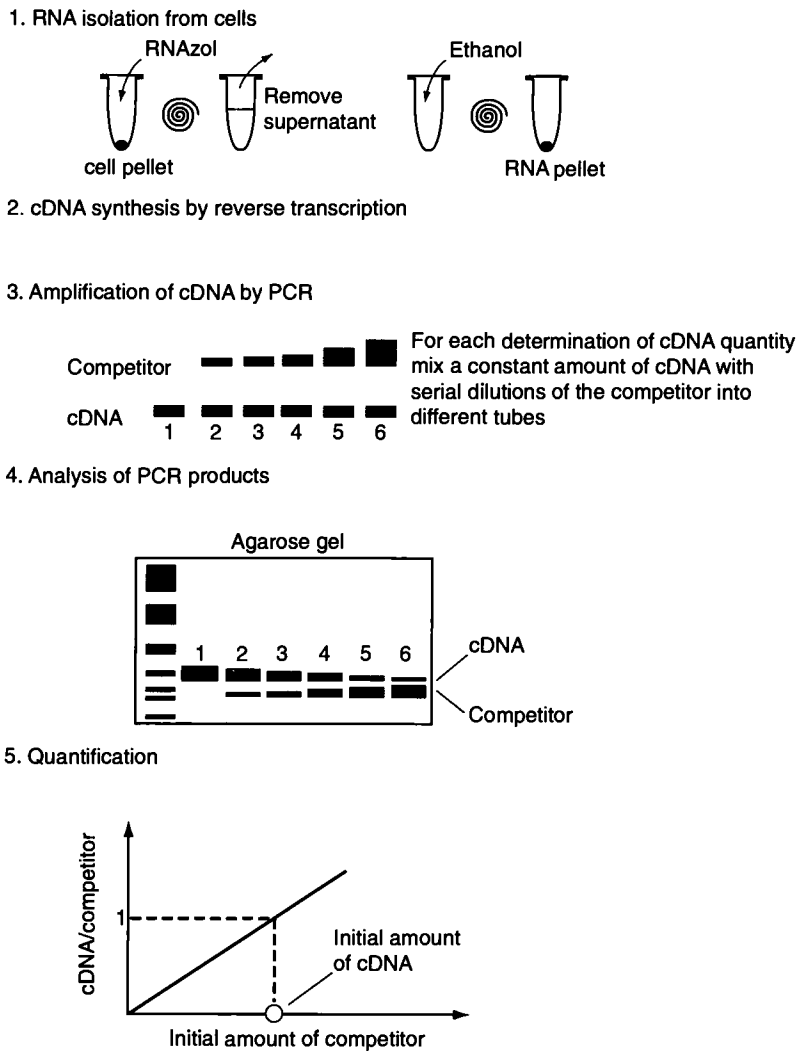

Figure 3. Competitive RT-PCR analysis.

can be expressed as the increase over the lowest-reading sample, which is arbitrarily given a value of 1. The results are then expressed on a histogram.

Another attractive and elegant method, because of its great accuracy and sensitivity, is to use a DNA sequencer to analyse PCR products that have been labelled with different fluorochromes on their forward primer (Porcher *et al.*, 1992; Cottrez *et al.*, 1994). This method allows many different dyes to be used in a single lane, and thus increases the possible number of loadings per gel. The analysis is carried out using suitable software such as Genescan, which integrates the fluorescent peaks, as described above.

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6 Measuring Immune Responses *In Situ*: Immunohistology and *In Situ* Hybridization

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INTRODUCTION

The indirect immunofluorescence technique developed by Coons and Kaplan in 1950 was the first to describe the localization of antigen in tissues using fluorescently labelled antibody. Since then, considerable progress in immunohistochemical procedures has been made, most notably on the application of enzyme-labelled antibodies and signal enhancement. Enzyme coupled detection methods were developed primarily because of the limitations of fluorescence techniques, such as naturally occurring autofluorescence, the lack of permanence of the preparations (which tend to fade), the difficult correlation to morphological details and the need for expensive equipment. Of a variety of enzymes assayed, the enzymes of choice for direct and indirect procedures became horseradish peroxidase (PO) and calf intestinal alkaline phosphatase (AP) (Nakane and Pierce, 1967). The sensitivity of these immunoenzymatic detection methods was greatly enhanced by the development of the peroxidase anti-peroxidase (PAP) (Sternberger *et al.*, 1970) and the alkaline phosphatase anti-alkaline phosphatase (APAAP) methods (Cordell et al., 1984), which are based on the utilization of enzyme bound non-covalently to enzyme-specific antibodies.

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With the advent of monoclonal antibody production techniques developed by Köhler and Milstein in 1975, problems associated with polyclonal antisera could be overcome in immunochemical techniques. Serum-derived antibodies may react with many irrelevant antigens, sometimes making interpretation of results difficult. Monoclonal antibodies help to overcome these difficulties, since they recognize only one specific epitope. Due to the homogeneity of monoclonal antibodies, standardization from laboratory to laboratory has become possible. In addition, hybridoma cell lines provide an unlimited supply of antibodies, in contrast to the limited supply of a polyclonal antiserum.

Great effort was also put into making paraffin-embedded material more amenable to immunohistological techniques. Although formalin remains the most popular fixative used, this fixative is not always the best for preserving the antigenicity of tissues, due to the intermolecular cross-linking between formalin and proteins. Approaches to unmasking the antigenic sites hidden by cross-linked proteins have been to develop antibodies that can recognize formalin-resistant epitopes, to choose the correct fixative and optimize the duration of fixation, and the application of protease digestion, to name a few. A major advance in antigen retrieval was achieved by Shi *et al.* in 1991 based on microwave heating of tissue sections attached to microscope slides to temperatures up to 100°C in the presence of metal solutions. This resulted in the possibility of visualizing antigens that were otherwise undetectable in formalin-fixed, paraffin-embedded tissues, and also greatly simplified the method for antigen retrieval. In a study undertaken by Cattoretti *et al.* (1993), antigen retrieval techniques were optimized for a large number of antibodies to be used on formalin-fixed, paraffin-embedded tissue sections.

The methods described below are based on reports in the literature. They have been developed to yield optimal signal to background ratios and are used routinely in our laboratory with consistently good results.

◆◆◆◆◆◆ SPECIMEN PREPARATION

For immunoenzymatic techniques, cryostat preparations generally have a superior preservation of antigens over paraffin sections. Morphological details, however, are more readily destroyed in cryostat sections and the great majority of archival material accessible for immunostaining is from paraffin-embedded specimens. In the following, the emphasis is on the treatment of cryostat and paraffin sections prior to staining. For more details on paraffin embedding and sectioning, see Bullock and Petrusz (1982), Coligan (1991), Watkins (1989), Zeller (1989).

Paraffin Sections

As mentioned above, for antigen retrieval it may be necessary to try several approaches to preparing paraffin sections for immunoenzymatic staining.

For no treatment for antigen retrieval, routinely processed paraffin sections are dewaxed by submerging for 10 min in xylene followed by 10 min in acetone and 10 min in a 1 : 1 mixture of acetone and Tris buffered saline (TBS). The sections are kept in TBS until staining.

For microwave oven pretreatment, paraffin sections are deparaffinated for 10 min in xylene and rehydrated in a series of ethanols or acetones at 10-min intervals (100%, 70%, 40%, 0%). The slides are transferred to a plastic staining jar filled with 10 mM sodium citrate buffer, pH 6.0. The plastic staining jars are put in the microwave oven and heated for 5 min at 720 W. After 5 min it is essential that the staining jar is refilled with H_2O to prevent the specimens from drying out, before heating for another 5 min. The frequency of the heating steps depends on the fixation and embedding procedures performed previously and should be optimized. After the microwave treatment the slides are cooled in the staining jar for approximately 20 min at room temperature and washed briefly in TBS before proceeding with immunostaining.

For pressure cooker pretreatment, sections are dewaxed and hydrated as for microwave treatment. Using a normal household pressure cooker (with a 15 psi valve), boil 10 mM citric acid, pH 6.0, before adding the sections. Make up enough buffer to totally submerge the sections (approximately 2 l). Rinse the sections briefly in distilled water and place them into the boiling buffer. Close the lid and heat until the top pressure is reached, and boil the sections at this pressure. The boiling time must be optimized (approximately 1–10 min). After cooking, immediately cool the pressure cooker under cold running water; take extreme care at this step, and follow the instructions of the manufacturer precisely before opening the cooker. The sections are transferred immediately into cold running tap water before proceeding with the immunohistological staining.

Frozen Sections

Several steps in the preparation of frozen material are crucial for obtaining samples with well-preserved morphological structure and antigenicity. We have consistently obtained excellent results using the following procedure.

Fresh surgical specimens are submerged in flat-bottomed polyethylene tubes (catalogue No. 619-x, Brand Laboratory Equipment Manufacturers, Wertheim, Germany) filled with sterile physiological NaCl or PBS, snap-frozen in liquid nitrogen and stored at –70°C. If tissues have not been appropriately snap-frozen, ice crystals will be present in the tissue and cause artefactual staining along the fracture lines caused by these crystals. Tissues should always be maintained in a frozen state, since thawing and refreezing will result in extensive damage to the tissue and loss of antigenicity. Cryostat sections (4–5 µm) are air dried for 4–24 h and then fixed for 15–30 min in acetone followed by 15–30 min in chloroform. If the cryostat sections are not to be stained the following day, air dry for 4–24 h, fix for 10 min in acetone and store at –70°C. When needed, thaw the sections covered to prevent water condensation on the slides. Fix in acetone and chloroform as described above.

For cytospin specimens, 1×10^5 to 5×10^5 cells are applied per slide and centrifuged for 5 min at 220*g*. The cytospins are air dried as described above for 4–2 h and stored at –70°C. Before staining, samples are thawed and fixed solely in acetone for 10 min.

For some staining procedures, primarily those involving cytokines, the fixing of specimens with paraformaldehyde/saponin may be preferred (Sander *et al.*, 1991). Air-dried slides are fixed for 15 min in 4% paraformaldehyde in phosphate buffered saline (PBS) without NaCl (pH 7.4–7.6), followed by a 15-min treatment in 0.1% saponin/PBS to elute cholesterol from the membranes. Wash thoroughly with PBS before immunostaining.

◆◆◆◆◆ IMMUNOFLUORESCENCE DETECTION

Fluorochrome-labelled antibodies allow a high resolution to be obtained, since subcellular structures can be studied at magnifications beyond the limits of resolution of the light microscope. Because fluorochromes can be chosen that do not have overlapping emission spectra, double immunofluorescence permits the study of two different antigens in the same specimen, even if they have identical subcellular distributions, thus allowing co-localization studies (see Plate 9d–f). In addition, fluorochrome-labelled specimens may be analysed using confocal microscopy and imaging procedures.

Direct and Indirect Staining

Fluorescence staining can be performed using a primary antibody, which is conjugated with a fluorochrome (direct staining) or with an unlabelled primary, and a fluorochrome conjugated secondary antibody directed against the primary immunoglobulin. In both cases, the staining procedure can be performed in the following fashion (Fig. 1a,b).

The fixed specimen is incubated for 10 min in TBS/10% bovine serum albumin (BSA) to block unspecific binding. Wash twice with TBS before applying the directly labelled or unlabelled primary antibody. Incubate for 30 min and wash twice with TBS. When using a directly labelled antibody, proceed with the optional DNA staining and mounting before viewing under a fluorescence microscope. For the indirect detection procedure, continue with the incubation of the flourochrome conjugated secondary antibody for 30 min. For optional DNA staining of nuclei, wash the specimen twice and incubate for 10 min with the bis(benzimide) dye Hoechst 33342 (6 $\mu g\,ml^{-1}$) or 1 $\mu g\,ml^{-1}$ 4′,6-diamidino-2-phenylindole (DAPI). Wash twice in TBS and mount in 10 µl 1,4-diazabicyclo[2,2,2]-octane (DABCO) mounting and anti-fading solution (Johnson *et al.*, 1982) before viewing under a fluorescence microscope. The specimens can be stored for further viewing at 4°C for several months.

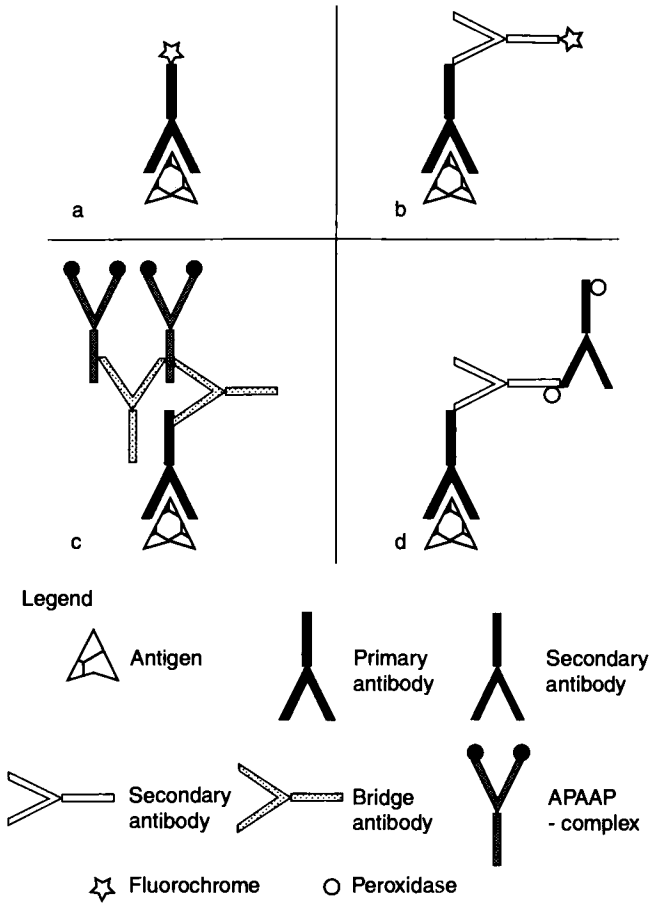

Figure 1. Scheme to show the principle of the methods described for immunofluorescence and immunoenzymatic detection of antigens. (a) Direct fluorescence, using a fluorochrome coupled primary antibody. (b) Indirect fluorescence with a fluorochrome coupled secondary antibody. (c) Alkaline phosphatase anti-alkaline phosphatase (APAAP) procedure, performed twice with the bridging antibody and the APAAP complex. (d) Indirect immunoperoxidase method using two different PO conjugated secondary antibodies.

Double Immunofluorescence Staining

For double immunofluorescence staining, the most common procedure is to use a combination of indirect and direct detection, using red and green fluorescing fluorochromes. The procedure is essentially as described above, with the following additional steps.

After the incubation for indirect detection (and before DNA counterstaining), the specimen should be blocked by a 30-min incubation with mouse serum (1:8 in TBS) when using anti-mouse secondary antibody, and mouse fluorescent labelled antibody for direct detection, or with other appropriate serum to block non-specific binding of the directly

conjugated antibody to the secondary antibody used for indirect detection. After washing twice in TBS, the directly conjugated antibody is added and incubated for 30 min. Following the washing procedure, continue as above for optional DNA staining and mounting with DABCO. Alternatively, a biotin-conjugated antibody can be used instead of a direct-conjugated antibody. The biotin-conjugated antibody can then be detected using fluorochrome-conjugated streptavidin or avidin.

◆◆◆◆◆◆ IMMUNOENZYMATIC DETECTION

The choice of the system to use (AP or PO) depends on several circumstances, such as the species in which the antigen is to be detected and the availability of appropriate primary and secondary antibodies, as well as the presence of non-inhibitable endogenous enzyme activity (e.g. intestinal AP is not inhibited by levamisol). For the APAAP procedure with human tissues, the primary antibody is usually a mouse monoclonal antibody. When using a rabbit antiserum an additional incubation step has to be included to make the antiserum detectable by the system.

In the following procedures, incubation steps are generally followed by washing twice in TBS. Slides are incubated with 100 µl antibody dilution for 30 min at room temperature in a level humid chamber. Excessive humidity should be avoided, since water condensation on the slides will interfere with the staining reaction. Insufficient humidity, on the other hand, will cause the antibody on the sections to dry, resulting in false-positive staining. Drying is usually most apparent at the edges of sections (rim effect).

Alkaline Phosphatase Anti-Alkaline Phosphatase Method

The APAAP method was proposed by Cordell *et al.* (1984). It is described here for human tissue and with reagents used routinely in our laboratory (Fig. 1c and Plate 9a, b). It must be emphasized that the dilutions given may not apply in all cases, and should be optimized before routine use.

Incubation for 30 min with the appropriately diluted primary antibody in 10% fetal calf serum (FCS)/TBS is applied to prepared cryosections and cytopreparations directly after the last fixation step and to paraffin sections after washing in TBS. For washing after the first incubation, the use of separate containers for different antibodies and negative controls is recommended.

If the primary antibody is a rabbit antiserum, the following incubation must be performed: 30 min with a monoclonal mouse anti-rabbit immunoglobulin (catalogue No. M 0737, DAKO Diagnostika GmbH, Hamburg, Germany).

The secondary (bridging) antibody (rabbit anti-mouse IgG H + L antiserum (catalogue No. Z 259, DAKO Diagnostika GmbH, Hamburg, Germany)) is diluted 1:20 in TBS, 1:8 in inactivated human serum and incubated for 30 min.

The APAAP complex (catalogue No. M 800, Dianova, Hamburg, Germany) is diluted 1 : 40 in TBS/FCS and incubated for 30 min.

The incubations with the secondary (bridging) antibody and the APAAP complex are repeated once for 15 min each and may be repeated *ad libitum* to increase the detection limit.

For the visualization of the staining, the following developing solution must be prepared freshly each time. Amounts are for one staining jar and the correct order of addition is essential to achieve a result.

- Solution A: 35 ml APAAP-buffer, 12.5 ml AP buffer, 20 mg levamisole.
- Solution B: dimethylformamide with 8.3% (w/v) naphthol–arsenic biphosphate.
- Solution C: 250 µl $NaNO_3$ solution with 100 µl Newfuchsin solution.

After 1 min of reaction time, 125 µl solution C is added to 47.5 ml solution A; then 300 µl solution B is added and the pH is adjusted to 8.8. The solution is filtered before adding the slides and incubating for 20 min on a shaker. Counterstaining is performed in haematoxylin for 90 s, the specimens are washed and left in tap water for 5 min before being mounted with prewarmed (56°C) Kaiser's glycerol gelatine.

Indirect Immunoperoxidase Method

The advantage of peroxidase staining is a greater variability and flexibility in the availability and combinations of antibody, as well as a less time-consuming procedure. Again, the procedure delineated here is for human tissue and a mouse or rabbit primary antibody (Fig. 1d and Plate 9c).

Before proceeding with primary antibody incubations, it may be advisable to block endogenous peroxidase activity in the tissue by preincubating the slides for 20 min in a light-protected staining jar with 1% H_2O_2 in TBS. The mouse (rabbit) primary antibody is applied in the appropriate dilution in TBS/10% FCS, and incubated for 30 min. The first secondary PO-conjugated goat anti-mouse (rabbit) antibody is applied diluted in TBS/10% FCS and inactivated human serum. The second secondary PO-conjugated rabbit anti goat is also incubated for 30 min. In contrast to the APAAP method, this procedure involves only two signal-enhancing steps due to an increase of background staining with further incubation steps.

For development, prepare the developing buffer as described below, and incubate the slides with 100 µl each. Incubate in the dark in the humid chamber for 3–15 min. The degree of development can be checked microscopically and the incubation stopped at the desired point. Slides are counterstained in haematoxylin and mounted as described for the APAAP procedure.

◆◆◆◆◆◆ IMMUNOENZYMATIC DETECTION METHODS IN *IN SITU* HYBRIDIZATION

In situ hybridization detection methods may become necessary for the mRNA detection of low level expressed proteins, for instance cytokines,

where immunohistological methods fail to detect a signal. In the following, two methods are described, one of which involves the use of chemically modified probes and one that uses digoxigenin labelled probes, both of which can subsequently be detected using enzyme-coupled antibodies against these modifications. In both cases, previous Zamboni fixation (Stefani *et al.*, 1967) of tissues and cells is recommended, since RNA is well conserved using this procedure. Samples are fixed for 10 min at room temperature in Zamboni's fixative immediately after preparation, washed extensively in 70% ethanol and stored in 70% ethanol at 4°C until further use. Because of the wide range of *in situ* hybridization procedures (hybridization solutions, washing procedures), the reader should consult the appropriate literature for further information. In the following, a hybridization and washing procedure is described which was used successfully for *in situ* detection of tumour necrosis factor α (TNFα) mRNA in cytocentrifuge prepared cells (Kretschmer *et al.*, 1990).

Sulfonated Probes

The generation of sulfonated probes is based on the chemical modification of cytidine residues internal to the probe (Budowsky *et al.*, 1972; Sverdlov *et al.*, 1974). Since only 10–15% of the cytidines in a given sequence are effectively modified, the sensitivity of the detection can be augmented by adding 12 cytidine residues to the 5'-end of oligonucleotide probes.

For the modification, add 25 µl solution A and 12.5 µl solution B (see Appendix to 10 µg denatured DNA in 50 µl H_2O and mix carefully. Incubate for 4 h at room temperature and store at –20°C until further use. The efficiency of sulfonation is confirmed by dot-blot analysis with immunoenzymatic detection of serial dilutions of labelled probes. For prehybridization, slides are transferred from 70% ethanol to PBS containing 5 mM $MgCl_2$ and incubated at room temperature for 10 min (repeat once). Incubate in deionized formamide (50% in 5× standard saline citrate (SSC) – see below under 'Chemicals and Solutions') and then prehybridize with 100 µl prehybridization solution per slide for 1 h at 37°C. Wash in deionized formamide (50% in 5× SSC) before hybridization for 2 h at 37°C. Slides are washed for 10 min in 1× SSC, 50% formamide in 1× SSC and 1× SSC before visualization. For visualization, slides are incubated with anti-sulfonated DNA antibody (Chemiprobe, Organics, Yarne, Israel) (Poverenny *et al.*, 1979) for 30 min, followed by the detection of bound antibody using the APAAP technique as described above.

Digoxigenin Labelled Riboprobes

The *in vitro* transcription of a desired DNA sequence cloned into the polylinker site of an appropriate transcription vector (e.g. pGEM3Z, Promega Biotech, Madison WI, USA) and flanked by promoters for SP6, T7 or T3 RNA polymerase can be used to generate digoxigenin (DIG) labelled sense and antisense riboprobes. Labelling is carried out using the

labelling kit according to the manufacturer's description (DIG RNA labelling kit (SP6/T7, catalogue No. 1175025, Boehringer Mannheim, Mannheim, Germany). Desired probes can also be labelled using polymerase chain reaction, nick translation, random primed labelling and 3'-end labelling (Boehringer Mannheim, nonradioactive *in situ* hybridization application manual). Efficiency of labelling is analysed using dot-blot hybridization, with subsequent immunoenzymatic detection.

Prehybridization and hybridization are performed essentially as described above, but with 5–10 ng ml^{-1} of probe. The detection is performed using AP- or PO-coupled anti-DIG antibodies (Boehringer Mannheim, Mannheim, Germany) with alkaline phosphatase or peroxidase detection, or with monoclonal anti-DIG antibody (Boehringer Mannheim, Mannheim, Germany) and detection of bound antibody using APAAP for signal amplification.

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APPENDIX

Antibodies

In general, care should be taken in choosing the correct combination of antibodies for the choice of detection system, i.e. compatibility of antibodies (isotype, species) and prevention of cross-reactivity (species from which the antibody originates, species of secondary antibodies, species for which the antigen is to be detected), and choice of serum used to block non-specific reactions. Each first antibody should be titred against a known tissue, such as normal lymph node or spleen, before it is used in the laboratory. Most antibodies are used in a concentration of 20–40 μg ml^{-1}, and most commercially available antibodies are used at titres of 1 : 20

to 1:200. In our experience, a first evaluation of the correct titre is obtained by using dilution steps of 1:10, 1:30, 1:100, 1:300, 1:1000 and 1:3000. For storage, sterility of antibodies should be maintained, and freezing and thawing should be avoided. Most antibodies are stable for months at 4°C.

Information on antibody specificity and source can be obtained in the literature (Schlossman *et al.*, 1995; *Proceedings (I–IV) of the International Workshop and Conference on White Cell Differentiation Antigens*; Weimer, 1995).

Chemicals and Solutions

Chemicals used for fixation procedures should be of p.a. grade.

- Tris buffered saline (TBS): 50 mM Tris (tris(hydroxymethyl)aminomethane), 150 mM, NaCl, pH 7.5.
- Phosphate buffered saline (PBS): 150 mM NaCl, 10 mM $NaH_2PO_4 \cdot xH_2O$, pH 7.2.
- Haematoxylin stock solution: 1 g haematoxylin, 0.2 g $NaIO_3$, 50 g aluminium potassium sulfate dodecahydrate ($KAl(SO_4)_2 \cdot 12H_2O$), 1000 ml H_2O. Finally, add 50 g chloral hydrate and 1 g citric acid.
- APAAP developing buffer: 1.21 g Tris, 5.85 g NaCl, 1000 ml H_2O.
- AP buffer: 12.1 g Tris, 5.85 g NaCl, 1000 ml H_2O.
- Newfuchsin solution: 5 g Newfuchsin in 100 ml of 2N HCl; store in a dark glass vessel at 4°C.
- $NaNO_3$ solution: 6% (w/v) in H_2O.
- PO developing buffer: dissolve 6 mg 3,3′-diaminobenzidinetetrahydrochloride in 10 ml TBS and mix with 100 µl H_2O_2 directly before use.
- DABCO anti-fading solution: dissolve 2.5% DABCO (1,4-diazabicyclo[2,2,2]octane) in 90% glycerol overnight. Adjust to pH 8.6 with 2 N HCl. Store at room temperature.
- Zamboni's fixative: 20 g paraformaldehyde is added to 150 ml saturated, aqueous picric acid solution filtered twice, heated to 60°C, and 2 N NaOH is added dropwise until the solution is clear. The cooled solution is filtered, made up to 1000 ml with 0.15 M PBS and stored at 4°C in the dark.
- Modification solutions for sulfonated probes:
- – solution A: 1 M methylbisulfite,
- – solution B: 1 M *O*-methylhydroxylamine
  The substances are dissolved in 10 ml H_2O and the pH is adjusted to 6.0 with 10 N NaOH. Stored at 4°C, the sterile filtered solutions are stable for approximately 10 weeks.
- Prehybridization solution: 500 µl deionized 100% formamide, 250 µl 20× SSC, 50 µl tRNA (10 mg ml^{-1}), 5 µl ficoll (40 mg ml^{-1}), 5 µl polyvinylpyrolidone (40 mg ml^{-1}), 5 µl bovine serum albumin (40 mg ml^{-1}), 10 µl 5 M NaH_2PO_4, 75 µl H_2O, and 100 ml denatured herring sperm DNA (1 mg ml^{-1}).
- Hybridization solution: 500 µl deionized formamide (100%), 150 µl 20× SSC, 25 µl tRNA (10 mg ml^{-1}). Mix 34 µl of this solution with 1–10 µl of sulfonated probe and adjust to a final volume of 50 µl with H_2O. Boil the solution for 2 min, add 1 µl Denhardt's solution (50×) and transfer to cytospin preparations.
- 20× SSC: 3 M NaCl, 0.3 M sodium citrate; pH 7.0.

Controls

Immunoenzymatic staining

Negative controls should include an unrelated antibody of the same immunoglobulin class at an equivalent concentration (isotype control). The negativity of secondary reagents is confirmed by incubating a sample without the primary antibody (TBS control).

Positive controls should be included where possible in order to exclude negative results due to incorrect staining procedures. Specificity of the primary antibody can be analysed by neutralization experiments, where blocking of antibody binding results in negative staining. This is done prior to the application in immunoenzymatic staining, by preincubating the antibody with recombinant or purified antigen for 30 min at 37°C.

In situ hybridization

Negative controls should include the hybridization with the sense probe. Specificity can also be investigated by applying RNase digestion to the specimen before hybridization procedures. In analogy, RNase digestion after hybridization can be performed to reduce non-specific background staining due to non-specifically bound probe.

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7 Isolation, Characterization and Cultivation of Human Monocytes and Macrophages

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CONTENTS

Introduction
Isolation of peripheral blood monocytes
Culture of human monocytes
Characterization of monocytes and monocyte-derived macrophages

INTRODUCTION

Monocytes (MOs) and the different types of tissue macrophages (MACs) are grouped together as the 'mononuclear phagocyte system' (MPS). As described for other species, it is assumed that mature human tissue MACs arise from circulating blood MOs upon leaving the vasculature (van Furth, 1989). The circulating pool of MOs is supplied by proliferating progenitor cells in the bone marrow. The proliferative capacity of MOs and tissue MACs is quite low, although some proliferation may contribute to cell homoeostasis in different tissues. The cells of the MPS have a prominent role in host defence against microbial pathogens and malignant cells, due to their capacity of phagocytosis, secretion of cytokines, enzymes, oxygen radicals and other soluble products, and direct cellular cytotoxicity. Furthermore, they have an important position at the bridge between innate and specific immunity in presenting antigen to T-cells in the context of major histocompatibility complex (MHC) class II antigens. In this respect, however, MOs and classical (exudate-type) MACs are inferior to the specialized population of dendritic cells, at least in stimulating naive T cells (Steinman, 1991). In the last few years, the differentiation of dendritic cells from MOs has been described, indicating a close connection between dendritic cells and the MPS (Peters *et al.*, 1996). As a separate chapter in this book is dedicated to human dendritic cells (see Chapter III.1), the aspects

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of dendritic cell differentiation and antigen presentation are not covered here. Rather, this chapter focuses on the classical functions of MOs and MACs.

In experiments with mice, peritoneal or spleen MACs are usually used as prototype members of the MPS. In the human system, blood MOs are the starting cell population that is most easily obtained. These cells can be cultured *in vitro* in order to obtain prototypic 'tissue' or 'exudate-type' MACs (Musson *et al.*, 1980; Andreesen *et al.*, 1983b), as described below. Researchers in hospital departments may sometimes have access to clinical specimens of bronchial lavage that contain alveolar MACs or specimens of peritoneal dialysis fluid that contains peritoneal MACs. MACs in solid tissues can be detected by immunohistochemistry, as is described elsewhere (see Chapter III.6). Fresh tissue samples can also be processed in order to isolate MACs for functional studies; this will not be discussed here, because few researchers have regular access to fresh human tissue (see Chapter II.2.5 for some general techniques).

◆◆◆◆◆◆ ISOLATION OF PERIPHERAL BLOOD MONOCYTES

Human blood MOs are usually purified from mononuclear cells (MNCs) prepared by standard density centrifugation over Ficoll/Hypaque (see page 540). MNCs can be purified from blood, from buffy coats or from white cell products obtained by leukaphoresis. A leukaphoresis procedure is the optimal method for obtaining large numbers of cells. If you have access to this method in a blood banking or haematology department, attention must be paid to the fact that standard settings for harvesting of lymphocytes or peripheral blood progenitor cells will only lead to low MO yields. High numbers of MOs will be obtained if cells are collected deep within the interface which contains rather high numbers of red blood cells) (Brugger *et al.*, 1991c). With appropriate settings, a leukaphoresis product containing 5×10^9 to 8×10^9 MNCs can be expected from a healthy donor, with 10^9 of these cells being MOs. If in doubt, contact the supplier of your aphoresis system. In many laboratories, buffy coats from local blood bank services are used as starting material. These should be freshly prepared, a requirement that quite frequently leads to logistical problems. If preparation of MOs from buffy coats gives low yields or if other technical problems occur, freshly drawn blood anticoagulated with preservative-free heparin should be tried as a starting material for comparison.

MOs can be obtained from MNC preparations by several different methods. Sorting by fluorescence-activated cell sorting (FACS) or magnetic beads is described elsewhere in this book (see Chapter I.1). Density gradient centrifugation (e.g. with percoll) is another possibility (Davies and Lloyd, 1989), although the MOs will be contaminated by platelets that have to be removed in an additional adherence step. Two methods are described here in detail: (1) separation by cell adherence, which is simple and does not require expensive equipment; and (2) countercurrent elutria-

tion, which is the technique of choice in laboratories where an elutriation centrifuge is available and large numbers of cells have to be processed or preactivation of MOs by adherence (Haskill *et al.*, 1988) has to be avoided.

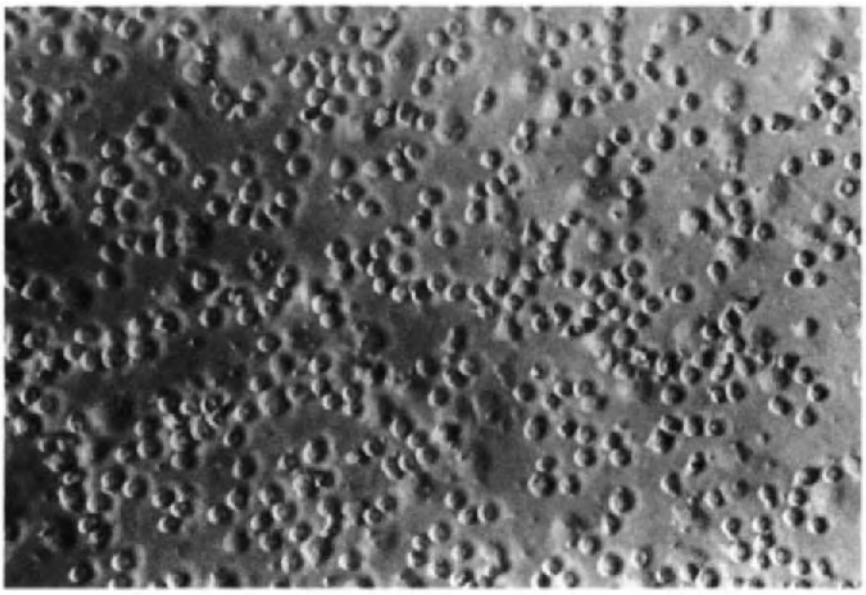
If large numbers of cells are needed, permanent cell lines may also be considered. Of course, these permanently dividing cells of malignant origin are in many ways quite different from normal human MOs or MACs; however, they may be suitable, depending on the experiments that are intended. Among the many monocytic cell lines, two seem to share quite a number of features with MOs or MACs: MonoMac 6 (Ziegler Heitbrock *et al.*, 1988) is $CD14^+$ and morphologically monocytic; and THP1 cells (obtainable from American type culture collection (ATCC)) are carboxypeptidase M positive and transform to morphologically MAC-like cells when stimulated by phorbol esters.

Purification of Monocytes by Cell Adherence

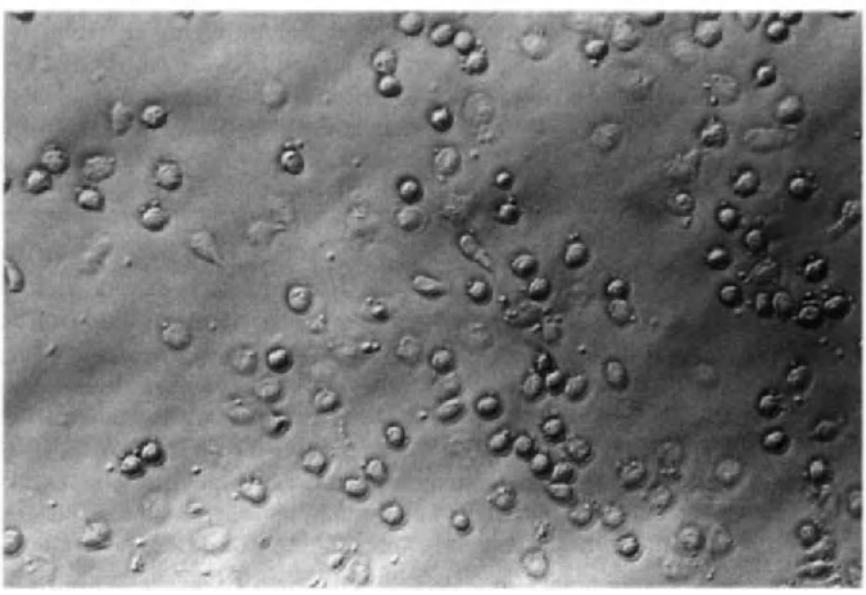
MOs and MACs have an intrinsic avidity to adhere to cell culture plastic or glass surfaces. They will attach to 'bacterial grade' or 'tissue-culture grade' polystyrene, somewhat more weakly to Teflon, but not or only very weakly to polypropylene or polyethylene. This avidity for cell adhesion can be used to separate MOs and MACs from other cells. The adhesion of MOs and MACs is different from that of fibroblasts or cancer cell lines. It occurs within a few minutes at 37°C, but not in the cold, and it cannot be reversed by trypsination.

Standard protocol

1. MNCs are prepared by Ficoll/Hypaque density centrifugation (see Chapter II.2). After three washing steps in phosphate buffered saline (PBS), they are resuspended in cell culture medium containing 2% human serum at a density of 5×10^6 MNC ml^{-1}. Keep in the cold.
2. *Complete medium.* RPMI-1640 with 2% human serum, $NaHCO_3$, 2 mM glutamine, 50 μM 2-mercaptoethanol, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycine, minimal essential medium (MEM) non-essential amino acids, MEM vitamins, MEM pyruvate. (MEM supplements are diluted 1/100 from 100× stock solutions; Gibco-BRL, Paisley, UK).
3. About 3 ml of cell suspension (15×10^6 MNCs) containing an estimated 3×10^6 MOs are pipetted into a 60-mm Petri dish with a hydrophilic Teflon surface (e.g. Petriperm, Heraeus, Osterode, Germany) and placed in an incubator at 37°C, 7% CO_2. At the same time, prepare about 20 ml complete medium prewarmed to 37°C. After 30–45 min, attachment of MOs to the surface of the culture vessel can be examined under an inverted microscope (Fig. 1A). If insufficient adherence occurs, the incubation time can be extended up to 90 min, but this is not usually necessary. After incubation, swirl the plates very gently to suspend the non-adherent cells, and suck off the cell suspension, which contains mainly lymphocytes and platelets. Rinse the plates gently with 3 ml prewarmed medium, taking care not to flush away attached MOs by the fluid jet from the pipette.

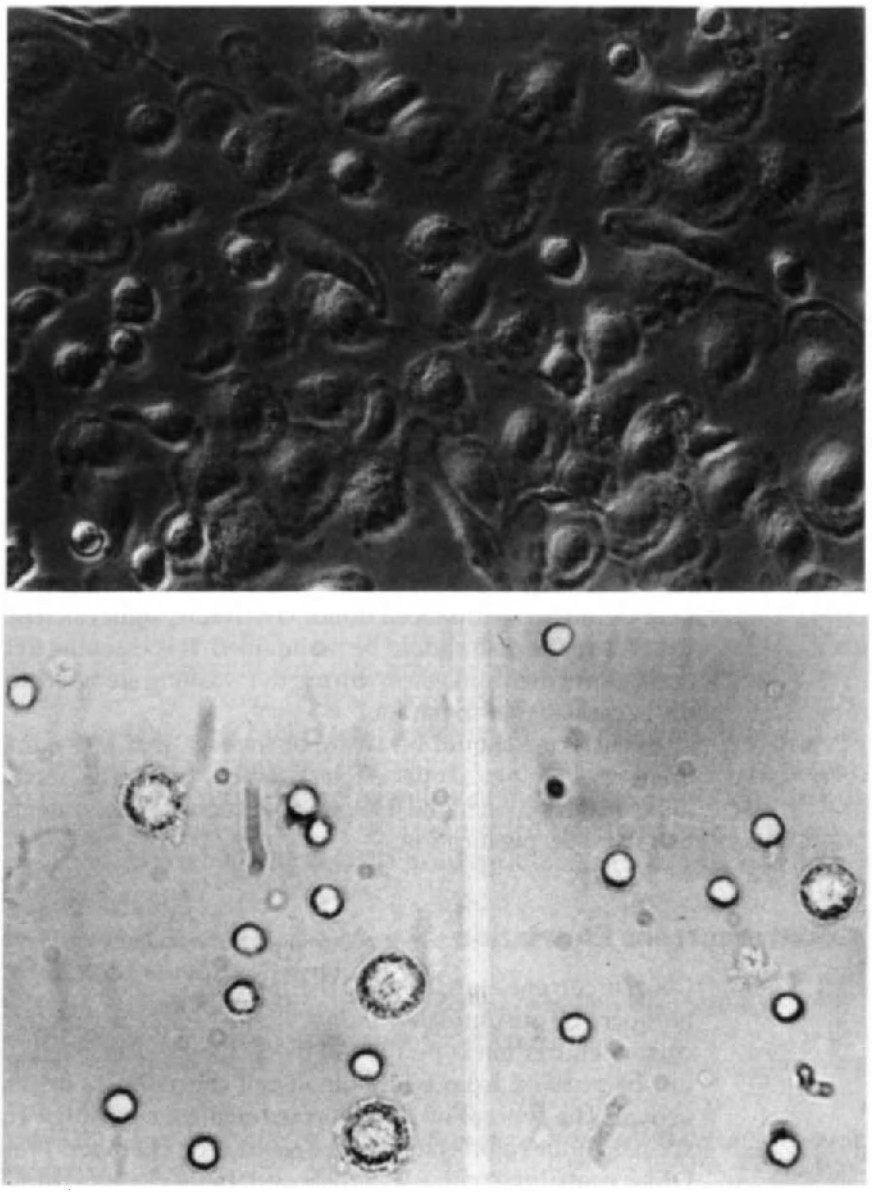


A



B

Swirl gently and suck off the cell suspension. Repeat this procedure twice. After three washing steps, cells are examined under the microscope (Fig. 1B). If many non-adherent cells are still present, the procedure can be repeated once or twice. When sufficient purity has been achieved, add 3 ml medium or PBS and place the dish on ice for 1 h in order to detach the cells. Subsequently, MOs can be harvested from the dishes by vigorous pipetting, counted in Trypan Blue and used for further experiments. Alternatively, MO-containing dishes can be incubated overnight in complete medium and harvested on the next day taking advantage of the fact that MO adhesion usually decreases slightly after 1 day of culture.



C

D

Figure 1. Phase contrast microscopy of MOs and MACs. (A) MNCs including adherent MOs in cell culture dishes. (B) The same dishes as in (A) after washing away most of the non-adherent cells. (C) The same dishes after 1 week of culture: MAC differentiation has occurred, leading to much larger cells (MACs with a similar morphology will develop in Teflon bags). (D) A mixed population of MACs and lymphocytes harvested from a Teflon bag is placed in a chamber for cell counting. All figures: ×200.

Modifications of the protocol and comments

The protocol described above has the advantage that MOs can reliably be detached from the Teflon dishes for further experiments. A disadvantage of this method, compared to the use of polystyrene dishes, is that more MOs will be lost with the non-adherent cell fraction due to the less avid cell adhesion to Teflon surfaces. Furthermore, Teflon dishes are quite expensive. If polystyrene plates are used instead of Teflon, MOs will attach more tightly, but their removal for use in further experiments may be difficult. If subsequent experiments can be performed directly on the dishes without determination of the exact number of cells, the use of normal tissue culture plastic or bacterial grade plastic is preferable (see pages 670–671 for additional discussion of culture substrates). If cells are to be detached from normal plastic, we suggest overnight incubation of the washed MOs at 37°C in complete medium before putting them into the refrigerator or on ice and trying to detach them by vigorous pipetting. Some authors recommend the use of ethylenediaminetetraacetic acid (EDTA) for detaching adherent MOs, but in our experience this is not satisfactory. For unknown reasons, the strength of adhesion is also dependent on the individual cell donor. Therefore, tight microscopic control of the cells in the dish should be maintained. It is essential to keep the dishes and culture medium warm during the washing steps, so do not try to handle several dishes in parallel.

Finally, one should be aware of the fact, that MO adhesion induces a number of biological effects (Haskill *et al.*, 1988). This fact should be taken into consideration in the design of subsequent experiments, if adhesion is used for MO purification.

Countercurrent Elutriation

Countercurrent elutriation is the method of choice for separating large numbers of unstimulated monocytes. The starting material for countercurrent elutriation is peripheral blood mononuclear cells (MNCs), which can be purified from white blood cell concentrates or whole blood (see above). The separation is performed using a centrifuge equipped with a special elutor rotor system and a separation chamber. We use a Beckman J6M-E centrifuge with a JE-5 rotor system (Beckmann, Munich, Germany), which can be equipped with a small separation chamber for processing 10^8–10^9 MNCs or a large chamber for processing 10^9–10^{10} MNCs, in combination with a masterflex peristaltic pump (Barnant, Barrington, IL, USA). The principle of the separation is the balance between an outward-directed centrifugal force and inward-directed hydrodynamic force created by pumping fluid continously through the rotor in a centripetal direction (Fig. 2). While the rotor is spinning in the centrifuge, a suspension of cells is pumped at a preset flow rate from outside the centrifuge into the separation chamber. Cells migrate to positions in the separation chamber where both forces are balanced, i.e. they equilibrate at different radii due to their size and density (for details see Sanderson *et al.*, 1977). Different fractions of cells can be sequentially eluted from the centrifuge

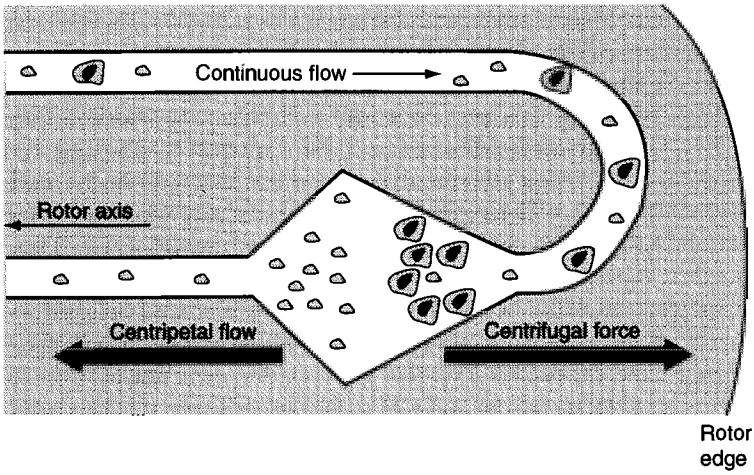


Figure 2. Principle of countercurrent elutriation of cells of different sizes. The smallest cells are washed out with the fluid flow, while the large cells are held back by the centrifugal force.

by increasing the fluid flow rate. Smaller cells (i.e. platelets, then lymphocytes) leave the system first, and monocytes, which are the largest cells, are washed out last (Table 1). Platelets and MOs can be obtained in rather high purity, whereas the cell distribution in the different lymphocyte fractions is widely overlapping. The purity of the monocyte fraction is about 90%. In general, about 90% of all cells loaded into the system are recovered, and the viability of the cells in all fractions is about 99% – as determined by Trypan Blue exclusion:

- Hank's balanced salt solution without Ca^{2+} and Mg^{2+}
- 6% H_2O_2 in sterile pyrogen-free water
- human serum albumin (clinical grade) or autologous human plasma
- 70% ethanol in pyrogen-free water.

Table 1. Separation of MNCs into different cell fractions by countercurrent elutriation*

Fraction	Flow rate (ml min^{-1}) Small chamber	Large chamber	Predominant cell type
I	52	12	Platelets, few small lymphocytes
II	57	13	Lymphocytes (many B cells)
III	64	14.5	Lymphocytes (mainly T cells)
IV	74	16.5	Lymphocytes (T cells, NK cells)
V	82	18	T cells, NK cells, monocytes
VI	111	25	Monocytes

* Settings are given for a Beckman J6M-E centrifuge with a JE-5 rotor system, using either a large or small separation chamber and a constant centrifuge speed of 2500 rpm.

Before each run, the separation chamber is assembled and sterilized by running about 500 ml of a 6% hydrogen peroxide solution through the system in a closed circuit for about 20 min. Avoid strong light exposure. During this procedure air bubbles may become trapped in the system. These air bubbles have to be removed by starting and slowing down the centrifuge several times (see the instruction manual for the system for details). The centrifuge is then set at the required speed (we use a speed of 2500 rpm, and all flow rates given here are for this speed) and kept at this speed throughout the whole procedure. Following sterilization, the system is flushed with 1000 ml sterile Hank's balanced salt solution or PBS to remove the hydrogen peroxide. Now the peristaltic pump system has to be calibrated. For this purpose the amount of fluid pumped through the system per minute at different settings of the peristaltic pump is measured and a calibration curve (pump setting versus fluid flow in ml min^{-1}) is drawn. The system is then flushed with Hank's solution supplemented with either 2% albumin or 6% human plasma. It is now ready for loading the MNC suspension. If the MNC preparation is not yet ready to be loaded, a closed circuit with the centrifuge and peristaltic pump running continuously can be installed.

The MNCs are loaded into the system at a flow rate of 52 ml min^{-1} for the large chamber. Small lymphocytes and platelets are removed at the same speed by collecting 1000 ml of fluid (see Table 1). By increasing the flow rate, cells of inceasing size are collected in fractions of 1000 ml. At a flow rate of 82 ml min^{-1} a mixed fraction (fraction V) of lymphocytes and monocytes is washed out. A higher flow rate for this fraction will give higher purity MOs in fraction VI, but at the expense of a considerable loss of MOs, because more MOs will be present in fraction V. Finally, at a flow of 111 ml min^{-1} the monocyte fraction (fraction VI) is obtained.

When all the desired cells have been collected, first stop the rotor and then the pump. The tubing should be flushed with sterile water followed by 70% isopropanol to remove all buffer, debris and remaining cells. The chamber must be disassembled, carefully cleaned and dried.

◆◆◆◆◆◆ CULTURE OF HUMAN MONOCYTES

Short-term Culture

The majority of MOs will survive for 1 day on a wide variety of culture substrates in different types of culture media. However, even if no dead cells are seen microscopically in MO cultures, this does not indicate that no cells are dying. The dead MOs are phagocytosed by their neighbours and, therefore, down to a survival of about 30%, the culture will still look 'healthy'. Overall, the researcher is quite free in his or her choice of culture system for a short-term analysis of MOs. Our standard cultures are grown in complete medium containing 2% human serum on Teflon or tissue culture plastic at 37°C and 7% CO_2. Serum-free conditions in standard

medium are also possible for short-term cultures. Endotoxin-free conditions are essential (see below for further discussion of this point).

As described above, MOs will adhere to almost any commonly used tissue culture laboratory ware or to 'bacterial grade' polystyrene. In fact it is almost impossible to cultivate MOs as non-adherent cells. They will attach to Teflon, albeit reversibly, and thus cannnot be called 'non-adherent' in this case, and if cultured on polypropylene surfaces they will form large clumps of cells sticking tightly together. However, it is possible to keep MOs alive and non-adherent in polypropylene tubes on a roller bottle device inside an incubator. For this purpose we use 200-ml polypropylene centrifuge bottles (Falcon/Becton Dickinson, Franklin Lakes, NJ, USA), which can be filled with about 40 ml culture medium and still placed in a horizontal position without spillage. The cap is placed loosely in position or, preferably, a ventilated cap from a culture flask is used instead. In this horizontal position, the bottles are rotated at about 0.2–1 rpm (we use the drive from the 'Miniperm' system (Heraeus, Osterode, Germany), but any other device that can be placed inside an incubator and keeps the bottle in a horizontal position while rotating is suitable).

Generation of Differentiated Macrophages in Long-term Culture

Mature human tissue MACs are not readily available for functional analysis. However, for many studies, *in vitro* MO-derived MACs can be used instead. If blood MOs are cultured *in vitro* in the presence of human serum, cells will increase in size and acquire some of the features of 'prototype' tissue or exudate MACs: the antigen phenotype will change (Andreesen *et al.*, 1990); the ability for phagocytosis, tumour cytotoxicity (Andreesen *et al.*, 1983a) and procoagulant activity (Scheibenbogen *et al.*, 1992) is increased; whereas the capacity to stimulate unprimed T-lymphocytes decreases (Schlesier *et al.*, 1994). Futhermore, the pattern of secretion products changes; for example, MOs produce large amounts of interleukin-1 (IL-1) upon stimulation, whereas mature MACs produce much more tumour necrosis factor (TNF) but no IL-1 (Scheibenbogen and Andreesen, 1991).

Standard protocol for MAC differentiation in Teflon bags

Rectangular bags are prepared in advance from Teflon foil (Biofolie 25, Heraeus, Osterode, Germany) by cutting pieces of appropriate size (e.g. 10×20 cm), folding once (with the hydrophobic side inside) and sealing on two sides with a heat-sealing device (e.g. Fermant 400, Joisten & Kettenbaum, Bergisch Gladbach, Germany) (resulting in bags with an inside size of about 3.5 × 18 cm). Use unpowdered gloves while preparing the bags. Appropriate settings of the sealing device have to be determined empirically. Test the quality of the sealing in a test bag with water before using the bags for cell culture. Sterilize in ethylene oxide.

Under a sterile hood, prepare a clamp with self-adhesive tape attached to a small stand to hold the Teflon bag, with the short, unsealed side

upwards (Fig. 3A). Prepare a suspension of 10^6 MO ml^{-1} in complete medium and fill the bags (10–20 ml is appropriate for a small bag such as that described above.) Seal the remaining side of the bag (Fig. 3B). Incubate at 37°C, 7% CO_2 for about 1 week. Refeeding is not necessary during a time period up to 7–10 days.

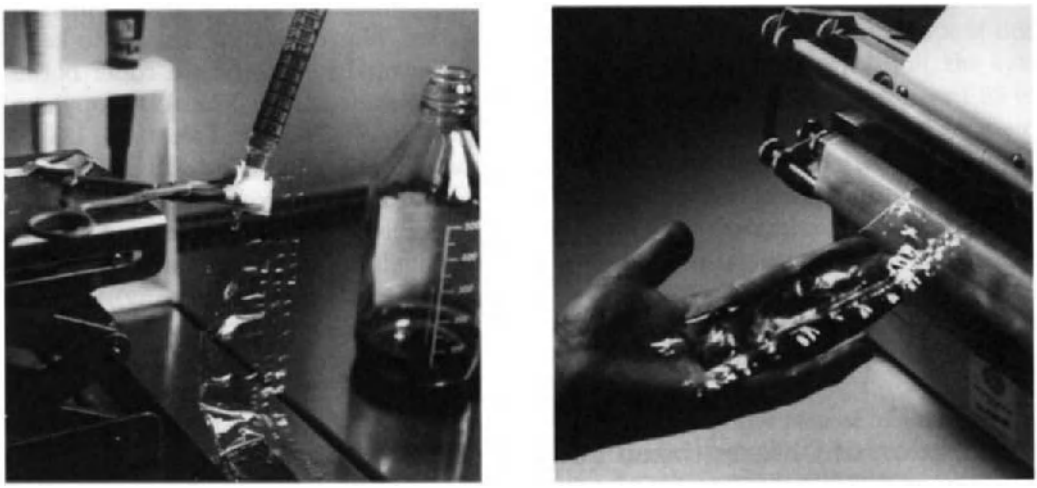


Figure 3. Teflon bags for long-term culture of MOs. (A) A bag is attached to a stand with self-adhesive tape and filled with cell suspension. (B) The bag is closed with a sealing device before incubation.

After an appropriate culture period, check under an inverted microscope whether MAC maturation has occurred (Fig. 1C). Teflon bags have to be placed on a thin transparent support tray (e.g. the lid of a multiwell plate) for microscopy. Place the bag at 4°C for 60 min, and then gently move the culture medium back and forth inside the bag with your fingertips in order to detach cells. Detachment of MACs can be seen macroscopically, but again this should be checked under an inverted microscope.

One corner of the bag is treated with 80% ethanol and cut off with scissors that have been sterilized over a gas flame. Transfer the contents of the bag to a polypropylene tube, wash once by centrifugation and count the cells by Trypan Blue exclusion. Differentiated MACs will be considerably larger (about two times the diameter) than contaminating lymphocytes which are also present (Fig. 1D).

Modifications of the protocol and comments

The protocol described above has several advantages: differentiated MACs can reliably be harvested, counted and transferred in defined cell numbers to subsequent experiments. Furthermore, the size of the Teflon bags can easily be scaled up for the processing of large cell numbers. For researchers who do not want to prepare their own bags, ready to use bags

can be obtained from commercial suppliers (e.g. DuPont, Wilmington, DE, USA). These bags do not need to be sealed, but can be closed with a Luer lock cap. Alternatively, 'petriperm' dishes (Heraeus, Osterode, Germany) can be used, but these are not as good as the bags (cells tend to stick firmly to the hydrophilic-type dishes, and maturation does not take place as well in the hydrophobic type).

MO to MAC differentiation will also occur on glass surfaces or on polystyrene. Any brand of laboratory equipment must be tested individually to see if it is suitable to support this process. In most instances, dishes specially prepared for tissue culture are inferior to standard 'bacterial grade' polystyrene. In our experience bacterial dishes or ELISA plates from Greiner (Frickenhausen, Germany) work quite well. If MO to MAC differentiation is followed microscopically daily, it will be seen that freshly plated MOs are adhered tightly. After 1–2 days, if the tissue culture substrate is not suitable, the adherence may decrease, and during the following days most cells will detach and subsequently die. On a 'good' substrate, MOs and MACs will initially attach firmly, and then spread out and increase in size daily. After 1 week differentiated MACs will be more strongly adhered than freshly isolated MOs (Fig. 1C). Therefore, in our experience, MO-derived MACs cannot be harvested reliably from any other substrate than Teflon. If seeded out again, MACs harvested from Teflon will tightly adhere to any tissue culture substrate, and contaminating lymphocytes or other non-adherent cells can easily be washed off without losing the MAC population.

Complete RPMI-1640 medium containing 2% human serum is our standard condition for differentiation of MOs from MACs *in vitro*. AB group serum is usually used, but this is not necessary, nor is heat inactivation of the serum. If significantly lower concentrations of serum are used, the full phenotypic change will not take place, while if higher concentrations of serum are used the MACs will acquire a somewhat rounder and more granular appearance, but the functional differences to MACs from 2% serum cultures are small. No specific growth factors have to be added, but autocrine secretion of macrophage colony stimulating factor (M-CSF) takes place and is important for MAC survival and differentiation (Brugger *et al.*, 1991a). Other factors can of course modulate the differentiation process. An obvious example is MOs in cultures containing IL-4 plus granulocyte–macrophage colony-stimulating factor (GM-CSF) and fetal calf serum (FCS) instead of human serum: in these cultures MO descendants with the typical phenotype of dendritic cells will develop (see Chapter III.1). Contaminating lymphocytes or platelets from MNC preparations do not severely affect normal MO to MAC differentiation, as long as the contaminating cells remain unstimulated. Therefore it is still possible to separate MACs from other cells after a 7-day culture of MNCs.

Note on Contamination by LPS or other Agents

Human MOs and MACs are extremely sensitive responders to stimulation by even minute amounts of endotoxin from Gram-negative bacteria,

i.e. lipopolysaccharide (LPS). A few picograms of LPS per millilitre may be sufficient to obtain a significant biological response, as for example characterized by the production of IL-1, IL-6 or TNF. It has even been suggested that this response could be used as a sensitive assay system to detect LPS contamination (Northoff *et al.*, 1987). If LPS is present in the cultures, this will obscure the response of cells to intentional stimulation by LPS or other stimuli. Moreover, normal MO to MAC differentiation will be disturbed after LPS stimulation (Brugger *et al.*, 1991b) and MOs and MACs will not respond to a second stimulation even some days later (a phenomenon called 'endotoxin tolerance') (Ziegler Heitbrock *et al.*, 1995). Endotoxin is the most frequent unwanted stimulus in MO cultures. However, other contaminants can be a problem. Heat-killed micro-organisms, including Gram-positive bacteria, may still activate the cells. Furthermore, as MOs and MACs are potent phagocytosing cells, they may clear an unapparent infection by living bacteria in the cell culture, and at the same time become stimulated.

From a practical point of view we recommend the use of sterile disposable laboratory equipment for cell culture work with human MOs and MACs. Materials that must be reused should never be touched without unpowdered gloves. After careful cleaning glassware should be baked at 250°C rather than autoclaved. Cell culture media, media supplements and serum should be ordered endotoxin free. Furthermore, regular measurements of spontaneous cytokine secretion should be performed. Production of IL-6 or TNF in unstimulated MO cultures should be below 2–5% of cultures stimulated with 100 ng ml^{-1} LPS. Cultures with higher rates of 'spontaneous' secretion should be regarded as preactivated.

◆◆◆◆◆◆ CHARACTERIZATION OF MONOCYTES AND MONOCYTE-DERIVED MACROPHAGES

Surface Antigens

The expression of many cell surface antigens changes during differentiation from MOs to MACs and is modulated by the special environment *in vivo* or by culture conditions *in vitro*. Typically seen in most MO or MAC populations are CD11c, CD14, CD33 and HLA-DR, among many others. Surface markers present on mature MACs include carboxypeptidase M (detected by MAX.1 and MAX.11 antibodies) (Rehli *et al.*, 1995), MAX.3, CD16, CD52 and CD71. No single marker can 'define' a cell as being a MO or MAC, instead several features have to be considered together (Andreesen *et al.*, 1990).

MO or MO-derived MACs harvested from Teflon bags can be analysed by flow cytometry or fluorescence microscopy without any particular difficulties (see Chapter I.1). A higher background fluorescence is observed for MO and MAC compared with lymphocyte populations. MACs are quite large cells: in flow cytometry the forward and sideward scatter gates have to be set differently than for freshly prepared MOs. Be aware of the

fact that unfixed viable MACs will readily adhere to the walls of polystyrene tubes unless kept in the cold.

Cell ELISA

MOs or MACs as adherent cells are particularly suitable for analysis by cell ELISA (Andreesen *et al.*, 1988). The density of a given antigen on adherent cells in a microculture well is determined by an enzyme-coupled antibody sandwich with a soluble substrate. Of course, the average antigen density of the whole cell population is measured, but the method has the advantage of allowing a high throughput of different samples in a reasonable period of time.

MOs can be cultured directly in sterile 96-well ELISA plates (Greiner, Frickenhausen, Germany) for up to 10 days; alternatively, MOs or MACs can be placed on the plates just before analysis and allowed to adhere for 60 min. A reasonable number of cells is 3×10^4 to 1×10^5 MOs or MACs per well. If cells have been cultured in the plates for several days, do not use the outer rows of wells, but fill these with culture medium to minimize evaporation from the inner wells. Examine microscopically, to see whether the cells are adherent. Flick off the cell culture medium and gently tap the plate upside down onto a paper towel. Put the plate on ice and immediately add 50 µl of fixing reagent (0.05% glutaraldehyde, 1% w/v glucose in PBS, pH 7.8) per well. Store fixing reagent at 4°C.

Incubate on ice for 10 min (this fixation works well with most antibodies, but every antibody must be tested for this application). Wash three times with NKH buffer (2 mM HEPES pH 7.4, 8.0 g NaCl, 0.4 g KCl in 1000 ml water; make fresh using 1 M HEPES stock solution), either in an ELISA washer or by using a pipette to fill the wells and then flicking off the fluid. Tap on a paper towel.

Block non-specific binding by incubating for 15 min at room temperature with 50 µl blocking solution (NAG) containing 10% normal swine serum:

- *Gelatine stock solution*: dissolve 5 g gelatine in 100 ml water, cool to room temperature, bring pH to 7.4, add sodium azide to a final concentration of 0.1%.
- *Blocking solution (NAG)*: 0.1% sodium azide, 40 mM HEPES pH 8.0, 0.2% w/v bovine serum albumin (BSA), 0.2% gelatine in NKH buffer. Prepare fresh from stock solutions or store in aliquots at –20°C.
- *NAG with 10% normal swine serum* (Dako, Glostrup, Denmark): mix and centrifuge for 3 min at 2000g to remove debris; discard the pellet.

Dilute the mouse monoclonal antibodies (mAbs) of interest in NAG. About the same dilution is appropriate as used for immunofluorescence or immunocytochemistry. Suck off the NAG blocking solution from triplicate wells and add 30 µl diluted antibody per well. Leave one triplet of wells without specific mAbs or add an appropriate negative control mAb that is unreactive against human cells. Include at least one positive antibody such as anti-β_2-microglobulin (HLA class I invariant chain) that has

a strong expected reactivity. Incubate for 15–30 min at room temperature. Wash three times with NKH buffer. Incubate for 10 min at room temperature with 50 µl diluted rabbit anti-mouse (RaM) antibody:

- *RaM stock solution*: 20 µl RaM (Dako, Glostrup, Denmark), 50 µl normal human AB group serum, 1 ml NAG. Prepare at least 12 h in advance. Can be stored for 4 weeks at 4°C.
- *Diluted RaM antibody*: 100 µl stock solution, 100 µl normal swine serum, 800 µl NAG. Centrifuge at 2000*g* for 3 min, discard the pellet.

Wash three times with NKH buffer. Incubate for 30 min with 50 µl diluted peroxidase-coupled goat anti-rabbit (GaR) antibody:

- *GaR buffer*: dissolve 8 g NaCl, 0.2 g KH_2PO_4, 2.9 g Na_2HPO_4, 0.2 g KCl, 0.5 ml Tween 20, 20 ml of 5% gelatine stock solution, 150 ml glycerol in a final volume of 1000 ml water. Adjust pH to 7.9. Store in aliquots at –20°C.
- *Diluted GaR*: dilute horseradish-coupled GaR $F(ab)_2$ (Dianova, Hamburg, Germany) 1 : 4000 in GaR buffer. Make fresh shortly before use.

Wash plates four times with NKH. Add 100 µl chromogenic substrate to each well and incubate for about 12 min in the dark:

- *Substrate buffer*: dissolve 10.5 g citric acid × $1H_2O$ in 500 ml water. Dissolve 17.8 g $Na_2HPO_4 \times 2H_2O$ in 500 ml water. Add citric acid solution to phosphate solution until pH is 5.0. Store at 4°C.
- *Substrate*: dissolve 30 mg 1,2-diphenylendiamine dihydrochloride (Fluka 78440, Neu-Ulm, Germany) in 50 ml reagent buffer. Add 20 µl of a 30% H_2O_2 stock solution directly before use.

Stop the reaction by adding 50 µl of 1 M H_2SO_4 per well. Determine the optical density at 492 nm in an ELISA reader. If possible, use 620 nm as a reference wavelength. The optical density for an antigen with strong expression should be at about 2.0, and background values for negative-control wells should be below 0.1. When first setting up the technique, it might be useful to try several dilutions of both secondary antibodies, starting with the dilutions given above. Furthermore, a slight increase or decrease in the colour reaction time may be appropriate. For better comparison of different experiments, we routinely measure β_2-microglobulin as a positive control that is homogeneously expressed on different MOs and MACs and then calculate an antigen expression index (AEI) (Andreesen *et al.*, 1988):

$$\text{AEI} = 100 \times \frac{\text{Specific antigen} - \text{Background}}{\beta_2\text{-Microglobulin} - \text{Background}}$$

Phagocytosis

The ability of phagocytosis is a prominent feature of mononuclear phagocytes. Whereas many cells will endocytose fluids or take up very small particles, MOs and MACs are also able to ingest particles up to a size of several micrometres (inert material, micro-organisms, cell debris, dead cells). The uptake of particles is accelerated by opsonization (i.e. coating

with complement or immunoglobulin). Macrophage phagocytic receptors recognize complement component 3 (C3b and C3bi) and the Fc part of immunoglobulin (Ig). Many protocols exist to test phagocytosis *in vitro*. A very simple method is to add latex particles (e.g. Sigma LB-11, St Louis, MO, USA) to cell cultures and to examine phagocytosis microscopically. Alternatively, MOs or MACs harvested from bags can be incubated with latex beads in small quantities of medium, washed by centrifugation and analysed microscopically. Two protocols for quantitative evaluation of phagocytosis are given below.

Flow Cytometry of Fluorescent Microspheres

The protocol described here is a modified version of the technique first reported by Steinkamp *et al.* (1982). Prepare phagocytosis medium. Add 10 µl beads (about 1×10^8 to 2×10^8 particles) to 1 ml phagocytosis medium and incubate for 10 min at room temperature. Do not store these opsonized beads.

- *Latex beads*: fluoresbrite carboxylated YG beads, diameter 1.7 µm, (Polysciences, 400 Valley Road, Warrington, PA 18976, USA).
- *Phagocytosis medium*: RPMI-1640 containing 1% w/v BSA, 2% human serum, 20 mM HEPES, pH 7.2.

Spin down 10^6 MOs or MACs harvested from Teflon cultures in 2-ml microcentrifuge tubes. Resuspend the cell pellet in 300 µl phagocytosis medium containing beads. Incubate for 60 min at 37°C (agitate the tube every 10 min or place the tube on a slowly rotating device in order to keep the cells suspended). After incubation, add 1.5 ml cold PBS/EDTA.

- *PBS/EDTA*: PBS containing 10 mM EDTA and 0.1% sodium azide.
- BSA 2% w/v in PBS/EDTA.

Perform all the following steps on ice.

Carefully layer the sample on to a 3-ml cushion of 2% BSA in PBS/EDTA (w/v) in a 10-ml centrifuge tube. Centrifuge for 7 min at 150*g*, 4°C. A large part of the non-phagocytosed beads will remain at the interphase. Carefully suck off the fluid from the top and resuspend the cell pellet in PBS/EDTA. Wash twice in PBS/EDTA by centrifugation (counterstaining with a red fluorescent antibody can be done at this time, before fixation; see Chapter I.1 and the comments below). Resuspend in 500 µl formaldehyde solution and store at 4°C until flow cytometric evaluation.

- *Formaldehyde solution*: dissolve paraformaldehyde to a final concentration of 1% (w/v) in PBS at about 50°C. Adjust the pH to 7.2 with 1 M NaOH (paraformaldehyde will only go into solution while adding NaOH). Store at 4°C for a maximum of 2 weeks.

For flow cytometry, use the linear scale for forward scatter (FSC) and sideward scatter (SSC) and the logarithmic scale for green fluorescence. First adjust the FSC and SSC sensitivity using control cells without beads.

Control cells should appear in the lower quarter of the SSC range, because the SSC will increase after phagocytosis (Fig. 4a,b). Keep in mind the appropriate settings, and then measure a sample of beads only. With the settings adjusted for MOs or MACs, no fluorescence signal should be detected, because beads alone are below the FSC detection limit. If you increase the FSC sensitivity and decrease the detection threshold, a sharp peak indicating the fluorescent beads will appear (Fig. 4c). Adjust the green fluorescence sensitivity in order to place the peak between 10 and 50 on a 10^4 scale. Store the measurement of beads without cells in a list file in order to document the fluorescence intensity of beads alone. Keep the fluorescence sensitivity unchanged and return the FSC sensitivity and threshold to the settings determined before with cells only. Now only cells should be detected as events, because beads without cells are below the FSC threshold.

The fluorescence intensity of the cells will correlate with the number of beads ingested. Cells having ingested no beads at all, or one, two or three beads, can clearly be separated from each other (Fig. 4d). In higher fluorescence ranges, the number of ingested beads can be estimated by comparing the fluorescence level of a single bead with the fluorescence of the bead-containing cells, assuming an exact logarithmic scale of the flow cytometer. Although this will not be absolutely exact, the data are clearly adequate for comparative studies. A check should always be made to see that the results obtained by flow cytometry match those obtained on microscopical examination. A fluorescence microscope is not necessary, as beads can also easily be seen using phase contrast. If large amounts of protein are still present after the cells have been washed, the remaining beads may start to aggregate into small clumps. These clumps may then be above the FSC threshold and be falsely interpreted as phagocytosing cells by flow cytometry. This phenomenon can be avoided if cell preparations are washed sufficiently and measured without a long delay on the day of preparation.

Instead of a short incubation of harvested cells, MOs or MACs can be incubated with the beads in cell culture in Teflon bags or dishes for 24 h. If the bead/cell ratio is not too high (below 20:1), centrifugation over 2% BSA can be omitted and the cells can be washed directly in PBS/EDTA after harvesting.

If counterstaining of surface antigens with a phycoerythrin (PE)-labelled marker is intended, proper compensation of two-colour fluorescence (see Chapter I.1) may become a problem, as the fluorescence intensity of the beads is very high. Counterstaining works best if cells do not ingest too many particles (less than 10–15); if necessary, cut down the number of beads used in the reaction. Futhermore, choose the antigen to be analysed by counterstaining to be one that is expressed intensely. Unstained cells, PE-stained cells and phagocytosing cells must be prepared separately and then mixed for setting the compensation parameters, before measuring the samples containing PE-counterstained phagocytosing cells. The green fluorescence of beads will appear strongly in the red channel, and high compensation settings have to be used to compensate for this.

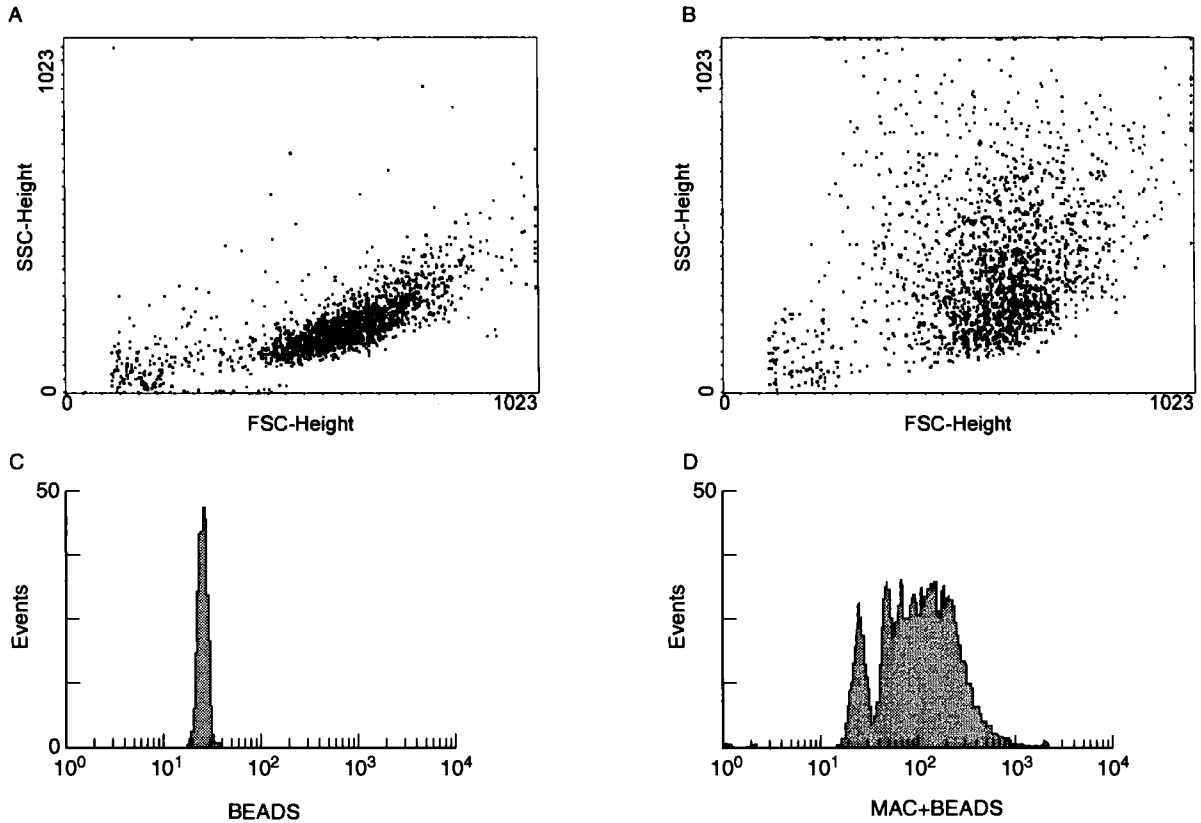


Figure 4. Flow cytometric evaluation of phagocytosis. (A) Scatter diagram for MACs harvested from Teflon bags. (B) Scatter diagram for MACs after phagocytosis. (C) Histogram for beads without cells. (D) Histogram for MACs with ingested beads.

Phagocytosis of immunoglobulin-opsonized sheep erythrocytes

Sheep erythrocytes can be coated with different ligands and therefore can be used to study the phagocytosis of opsonized particles by macrophages. A protocol for the quantitation of immunoglobulin-opsonized erythrocytes is given here.

Sheep red blood cells (10 ml, which is sufficient for a series of experiments) are washed three times with veronal-buffered saline/EDTA buffer. After incubating the cells at 37°C for 10 min, they are washed again twice in the same buffer. The buffer should be clear, with no detectable free haemaglobin.

- *Sheep red blood cells* (e.g. Behring, Marburg, Germany). These can be stored at 4°C for up to 2 weeks.
- *Veronal-buffered saline/EDTA buffer*: 4 mM NA-5,5-diethylbarbiturate, 20 mM EDTA, 0.121 M NaCl, 0.03% gelatine. Prepare from stock solutions and bring pH to 7.5.

Undiluted rabbit-anti-sheep haemolysin (50 µl) is added to the erythrocytes and the suspension is incubated for 20 min at 37°C with occasional agitation. After washing the erythrocytes three to four times in veronal-buffered saline/sucrose buffer, they are suspended at $5 \times 10^9 ml^{-1}$ in this buffer.

- *Rabbit-anti-sheep haemolysin* (e.g. S-1389, Sigma, St Louis, MO, USA).
- *Veronal-buffered saline/sucrose buffer*: 4 mM NA-5,5-diethylbarbiturate, 0.17 M sucrose, 0.15 M $CaCl_2$, 1 mM $MgCl_2$, 60 mM NaCl, 0.03% gelatine. Bring pH to 7.3.

Cells are plated into 24-well plates at a density of 2×10^5 MAC per well or 5×10^5 MO per well in 1 ml per well of RPMI-1640 with 2% human serum. Opsonized erythrocytes (10^8) are added to each well and the plates incubated for 2 h at 37°C. After this incubation period, the cells are washed three times with PBS, and the extent of phagocytosis can be examined under an inverted microcope. Externally attached erythrocytes are then lysed by immersing the plate in 250 ml distilled water for 15 s. The plates are washed three times in PBS. MOs and MACs are lysed with 1 ml of 0.01% digitonin solution (in distilled water) per well, and the samples are transferred to spectrophotometer cuvettes.

Negative controls are run in an identical fashion except that they are carried out on ice, so that phagocytosis should not occur. The number of erythrocytes phagocytosed is calculated by measuring spectrophotometrically the haemoglobin content of the lysed cells at 412 nm and subtracting the extinction values of the 0°C controls from those obtained at 37°C. As a control, 10^7 erythrocytes are lysed directly in 1 ml digitonin solution, and this value (expected extinction about 0.7) can be used for the quantitation of the absolute number of erythocytes phagocytosed in a specific test sample.

Oxygen Radical Formation

Monocytes/macrophages can destroy invading micro-organisms by reactive oxygen metabolites. The biochemical basis and key reaction for this

'respiratory burst' is the reduction of oxygen to O_2^- at the expense of NADPH by NADPH oxidase ($2O_2$ + NADPH → 2 O_2^- + $NADP^+$ + H^+). Hydrogen peroxide (H_2O_2) and other toxic metabolites are synthesized in subsequent enzymatic reactions. The production of oxygen radicals can be determined by one of several methods, such as nitroblue tetrazolium reduction, Fe^{3+} cytochrome-*c* reduction or chemoluminescence. Here we give a short protocol based on lucigenin chemiluminescence. Lucigenin detects primarily O_2^-, but other substrates for chemiluminescence (e.g. luminol) that preferentially react with other oxygen metabolites can be used instead.

- *Lucigenin* (bis-*N*-methylacridinium nitrate) in PBS, 10 mM stock solution, store at 4°C.
- *PMA* (phorbol 12-myristate 13-acetate) in dimethylsulphoxide (DMSO), 0.1 mM stock solution. Keep at −20°C.

MOs and MACs are placed in duplicate into polystyrene tubes (or disposable cuvettes, depending on the type of chemiluminometer used, e.g. from Berthold/Wallac, Munich, Germany) at a density of 2×10^5 cells per tube in 100 µl cell culture medium. PMA (or another known stimulus for the respiratory burst, e.g. zymosan) is used to induce the respiratory burst. Dilute PMA 1 : 500 in PBS and place 100 µl of this solution in the test tubes (final concentration 10^{-7} M). To test the spontanous (background) O_2^- production, 100 µl PBS is added to control tubes. Incubate the cells for an appropriate time period at 37°C (maximal respiratory burst with PMA is detected after about 30 min in MOs and after about 60 min in MACs). Dilute the lucigenin stock solution 1 : 10 with PBS, and pipette 50 µl of this solution into the tubes (final concentration 0.2 mM). Determine the chemoluminescence using the chemiluminometer. If no chemiluminometer is available, a β-counter can be used instead, but this gives a considerably lower sensitivity. Lucigenin can also be added at the beginning of the procedure at the same time as the stimulus, and measurements can be repeated at several time points up to 2 h if an evaluation of the time course of O_2^- production is desired.

Cytotoxicity against Tumour Cells

MACs can kill tumour cells by direct cellular cytotoxicity or by the production of cytotoxic effector molecules. Furthermore, they can act as effector cells in antibody-dependent cytotoxicity (Andreesen *et al.*, 1983a; Munn *et al.*, 1990). On the other hand, MACs may help tumour cell growth by supporting angiogenesis or by secreting growth factors (see Mantovani *et al.* (1992) for further references). *In vitro*, interaction between tumour cells and MOs or MACs depends on the state of differentiation and activation of effector cells and on the properties of the tumour target (Andreesen *et al.*, 1983a). The myeloid cell line U937 and the bladder carcinoma line RT4 (both can be obtained from ATCC) are examples of targets that are sensitive to MAC cytotoxicity *in vitro*. Cellular cytotoxicity can be tested by a multitude of different methods. The chromium release

assay, which is used to measure T-cell or NK-cell mediated cytotoxicity (see Chapter III.4), is not very suitable for measuring MAC cytotoxicity, probably because this is slower and because MACs ingest whole damaged cells.

Suspected apoptotic cell death of tumour targets can be tested by means of a number of different assays. In our experience the TdT-mediated dUTP-X nick end labelling method (Boehringer, Mannheim, Germany) works quite well. Antibody-dependent cellular cytotoxicity leads to phagocytosis of antibody-opsonized tumour targets, and this can be examined *in vitro* by using cell–ELISA or flow cytometry. For further information, the reader is referred to the work by Munn and Cheung (1990).

Tumour cytotoxicity measured by post-labelling with [^{3}H]thymidine

A system that can easily be adapted for the evaluation of the effects exerted by MOs or MACs is a post-labelling assay measuring [^{3}H]thymidine incorporation into the tumour target. This test cannot distinguish between real target cell death and growth arrest, but it is a very simple screening assay for determining the overall impact of effector cells upon tumour cell growth. Tumour cell proliferation is measured with and without co-culture with MACs. Human MOs and MACs do not proliferate *in vitro*, and thus contribute almost no background. However, MOs and MACs will metabolize MTT (dimethylthiazoldiphenyltetrazolium bromide). Therefore, with the latter detection system MOs, MACs and tumour targets must be separated physically after co-culture, and this is only possible for non-adherent tumour cells.

The technical details of measuring thymidine incorporation (or MTT metabolism) are described elsewhere in this book (see Chapter I.2) and therefore only the principle of the assay is outlined here. Tumor cell targets (10^4 per well per 100 µl of the tumour cell line under evaluation, e.g. U937) are placed in a microtitre plate. MOs or MACs as effector cells are added in triplicate in serial dilutions from 10^3 to 10^5 cells per well per 100 µl. Control-well triplets are left without effector cells and without target cells. Effector cells can also be stimulated using γ-interferon (200 U ml^{-1}) or other MAC-activating agents (in which case the control targets should be treated with the same stimulus). After a co-culture period of 48 h, cells are pulsed with 0.5 µCi of [^{3}H]thymidine per well and harvested 4–16 h later (see Chapter I.2 for details). Growth inhibition (GI) is calculated as:

$$GI = 1 - \frac{\text{cpm(co-culture)} - \text{cpm(MAC only)}}{\text{cpm(tumour only)}}$$

If effects mediated by soluble factors are suspected, the target cells can be physically separated from the effector cells by means of cell-impermeable inserts (e.g. Nunc, Roskilde, Denmark). In this case the tumour cells must first be prepared in the microtitre plates, after which the inserts are put into place and then the MOs or MACs are added. Inserts can be removed before pulsing the tumor targets with [^{3}H]thymidine. Alternatively, MOs

and MACs can be stimulated alone and the cell-culture supernatants then sterile-filtered and transferred to the wells containing the target cells.

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Beads for phagocytosis experiments.

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